"Investigating the hazards posed by pristine and modified Copper Nanomaterials using in vitro screens combined with in vivo models"

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Abstract

Nanomaterial use has increased as the properties of materials at the nanoscale differ substantially from bulk materials. However, there is still a lack of information concerning their impact on human health and environment.

Part of this study investigated the hazard of copper oxide nanoparticles (CuO NPs), multi-wall carbon nanotubes, silicon dioxide, pigment Red 254 and cobalt coated tungsten carbide using an *in vitro* hepatocyte model (HepG2/C3A cell lines). Most of the work was then conducted on CuO NPs together with four *Safer by Design* modifications of the same NPs (either citrate, ascorbate, polyethylenimine (PEI) or polyvinylpyrrolidone coating) and on both copper amine and micronized copper formulations, investigating their potency as inhibitors of fungal growth and analysing the gene expression *in vivo* on inhalation and ingestion models (Wistar rats).

Copper materials consistently exerted cytotoxicity to HepG2/C3A cells and induced cytokine production 24 hours post exposure. Micronized copper and ascorbate coated CuO were the most and the least toxic respectively.

Copper amine was most effective at reducing the fungal growth of *Coniophora puteana*, *Trametes versicolor* and *Gloeophyllum trabeum* while ascorbate coating enhanced the antifungal effect of CuO NPs.

In vivo, short-term inhalation studies (STIS) were performed with pristine, ascorbate and PEI coatings. All the materials upregulated tumour necrosis factor alpha (TNFα) in lung tissue; in the short-term oral study (STOS) with CuO NPs and micronized copper, only the latter downregulated chemokine C-X-C motif ligand 2 (CXCL2) in the liver and metallothionein 1A in the ileum.

In conclusion, CuO NPs were relatively cytotoxic *in vitro* and induced pro-inflammatory responses both *in vitro* and *in vivo*. However, these NPs were ineffective as antifungal treatment while the ascorbate coating enhanced the antifungal effect together with a significant decrease of cytotoxicity *in vitro*. The other materials did not induce any significant cytotoxicity nor cytokine production in C3A cells.

This work is dedicated to my wife Roberta who supported me the most through the entire length of the project and who is too modest to admit it.

To my son Ian who tried every strategy that a few month-old baby can use to prevent me to focus on this thesis to be cuddled instead.

To everyone who doubted me, *may you live in interesting times* (Terry Pratchett, 1994).

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"O frati," dissi, "che per cento milia
perigli siete giunti a l'occidente,
a questa tanto picciola vigilia

d'i nostri sensi ch'è del rimanente
non vogliate negar l'esperïenza,
di retro al sol, del mondo sanza gente.

Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza".

Dante Alighieri (1265-1321)
La Divina Commedia
Inferno: C. XXVI, vv. 112-120

‘O brothers!’ I began, ‘who to the west
through perils without number now have reach’d;
to this the short remaining watch, that yet

our senses have to wake,
refuse not proof of the unpeopled world,
following the track of Phoebus.

Call to mind from whence ye sprang:
ye were not form’d to live the life of brutes,
but virtue to pursue and knowledge high’.

Dante Alighieri (1265-1321)
The Divine Comedy
The Harvard Classics. 1909–14.
Inferno (Hell): C. XXVI, vv. 112-120

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Characterization of NPs and NMs

Both the report on the characterisation of pristine nanomaterials for (eco)toxicological testing and the report on the characterisation of NOAA in biological samples from (eco)toxicity tests are available online at the following links:

http://www.sun-fp7.eu/wp-content/uploads/2017/01/SUN_Deliverable_1.4.pdf

http://www.sun-fp7.eu/wp-content/uploads/2017/01/SUN_Deliverable_1_5.pdf

Deliverable D 6.1 - Report on non-immune and immune cell responses to the SUN priority NOAA in terms of the acute cytotoxicity, inflammatory and genotoxic effects

Endotoxin (LPS) detection

The procedure followed the Nanotechnology Characterization Laboratory protocol (NCL Method STE-1.1) using the assay QCL-1000™ Endpoint Chromogenic LAL Assay (Lonza). All values obtained were below 0.5 EU/ml (Figure A.1), considered the maximum admissible limit for medical devices. Additionally, no interferences between the NM samples and the assay substrate were observed.

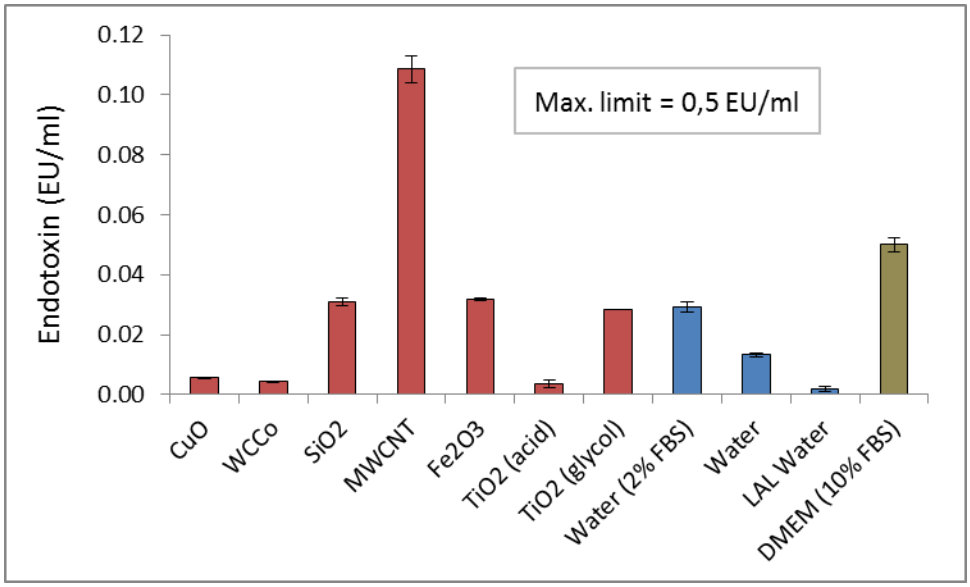


Figure A. 1 Evaluation of LPS contamination by LAL assay. The concentration tested was 1 mg/ml for all NPs, except MWCNTs (0.25 mg/ml), TiO_2 acid (0.06%) and TiO_2 glycol (0.12%).

Cytotoxicity

The cytotoxic potential of the nanomaterials was investigated using the Alamar Blue cell viability assay. Cells were exposed to increasing concentration of the nanomaterials (from 0 µg/ml to 125 or 200 µg/ml) for 24h; the MWCNTs were tested up to 48h for macrophages. The results raw data were collected using the SUN Excel templates and were used to calculate the benchmark dose 20 (BMD_{20}) and the EC_{50} for each NM by PROAST software.

For the macrophage cell line, the cytotoxicity assessment showed a high toxicity (BMD_{20} ≤100 µg/ml) for CuO, WCCo and SiO_2, and a low toxicity (BMD_{20} >100 µg/ml) for MWCNTs, the pigment NMs and TiO_2 (Table A.1 and Figure A.2). The most toxic NM was, however, CuO with the value of BMD_{20}=25.50 µg/ml and EC_{50}=40.97 µg/ml. Additionally, the salt forms of Cu and Co, $CuCl_2$ and $CoCl_2$ respectively were also tested using the same experimental design (Table A.1 and Figure A.3).

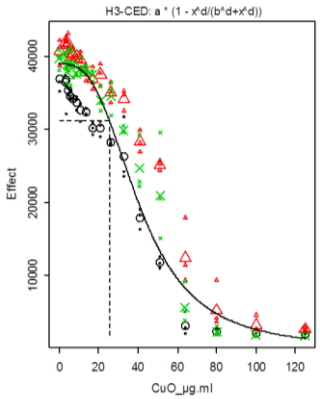
Table A. 1 NPs and NMs cytotoxicity on macrophage.

NOAA	**Time point**	**BMD_{20}** (µg/ml)	**EC_{50}** (µg/ml)
CuO_1_NP_SYN	24h	25.50	40.97
$CuCl_2$	24h	55.60	63.91

WCCo_1_NP_SYN	24h	48.10	98.08
$CoCl_2$	24h	32.70	58.54
MWCNT_1_NP_SYN	24h	132	>200
MWCNT_1_NP_SYN	48h	>200	>200
Pigment_1_NP_SYN	24h	123	>125
Fe2O3_1_NP_SYN	24h	>125	>125
SiO2_1_NP_SYN	24h	63.70	103.3
TiO2_1_SOL_MPG_SYN	24h	>200	>200
TiO2_2_SOL_HCl_SYN	24h	>200	>200

CuO_1_NP_SYN

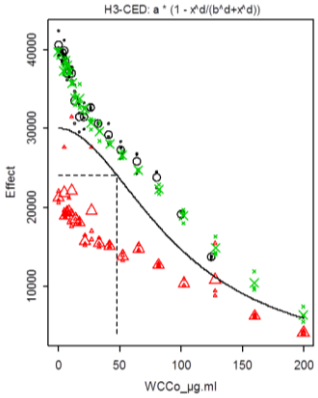
(24h)



A

WCCo_1_NP_SYN

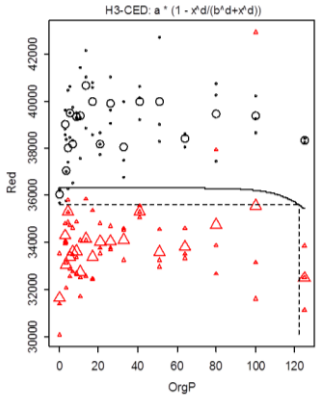
(24h)



B

Pigment_1_NP_SYN

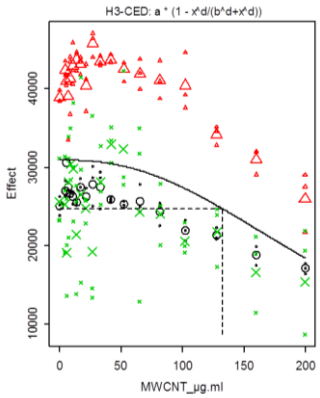
(24h)



C

MWCNT_1_NP_SYN

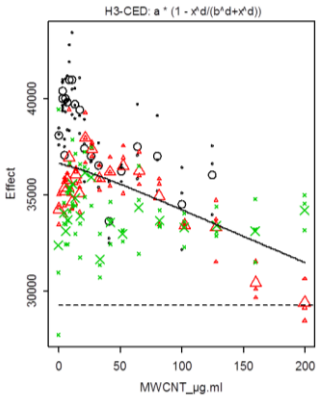
(24h)



D

MWCNT_1_NP_SYN

(48h)



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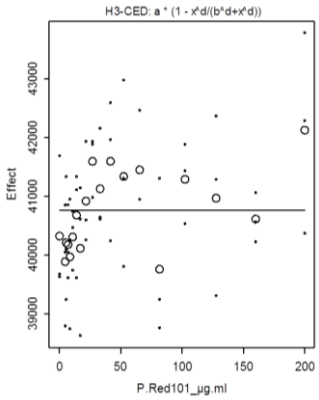
Fe2O3_1_NP_SYN

(24h)



F

SiO2_1_NP_SYN

(24h)

TiO2_1_SOL_MPG_SYN

(24h)

TiO2_2_SOL_HCl_SYN

(24h)

Figure A. 2 Representation of macrophage cell line cytotoxicity results and BMD_{20} calculation for the nanomaterials tested.

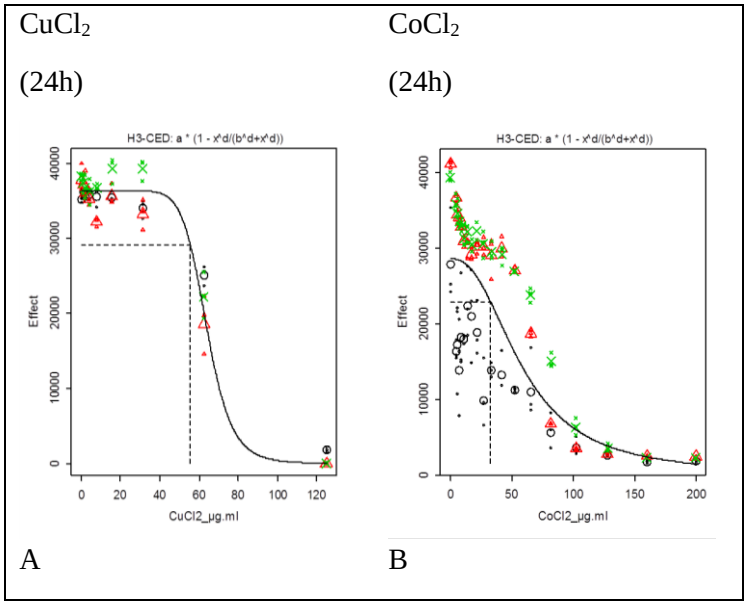


Figure A. 3 Cytotoxicity of the soluble forms of Cu and Co on macrophages.

Cytokine release

The inflammatory response was evaluated using the Luminex assay in the supernatant of cells exposed to CuO, WCCo and MWCNTs. The measurements were performed after 24h of exposure to concentrations corresponding to BMD_{20}x0.5, BMD_{20}x1, and BMD_{20}x2 except for MWCNTs which were tested only at the concentration of BMD_{20}x0.5 and BMD_{20}x1. The experimental setup included negative controls (culture medium only) and positive controls (cells exposed to 100 ng/ml of lipopolysaccharide, LPS). The possible interference of NMs with the cytokine detection was also evaluated.

The selection of cytokines to be analyzed was based on previous *in vivo* data (provided by RIVM), in-house experience with similar studies and a literature search. Ten cytokines and chemokines were selected: TNF-α, IL-6, IL-1β, IL-10, IL-12, MCP-1 (MCAF; CCL2), MIP-1β (CCL3), MIP-1α (CCL4), RANTES (CCL5), KC (IL-8, CXCL8).

The measurements in the supernatant after 24h of exposure to CuO showed an increase of TNF-α and MCP-1 while the other cytokines were decreased compared with the control (Figure A.4). The exposure to WCCo induced an increase of TNF-α, IL-6 and IL-12 and a decrease of the other cytokines comparing with the control (Figure A.4). Two cytokines analyzed (KC and IL-1β) could not be detected in any of the samples. The preliminary results obtained for MWCNTs showed an increase of the chemokine analyzed (MCP-1, MIP-1β and MIP-1α) but these need to be confirmed by additional measurements (data not shown).

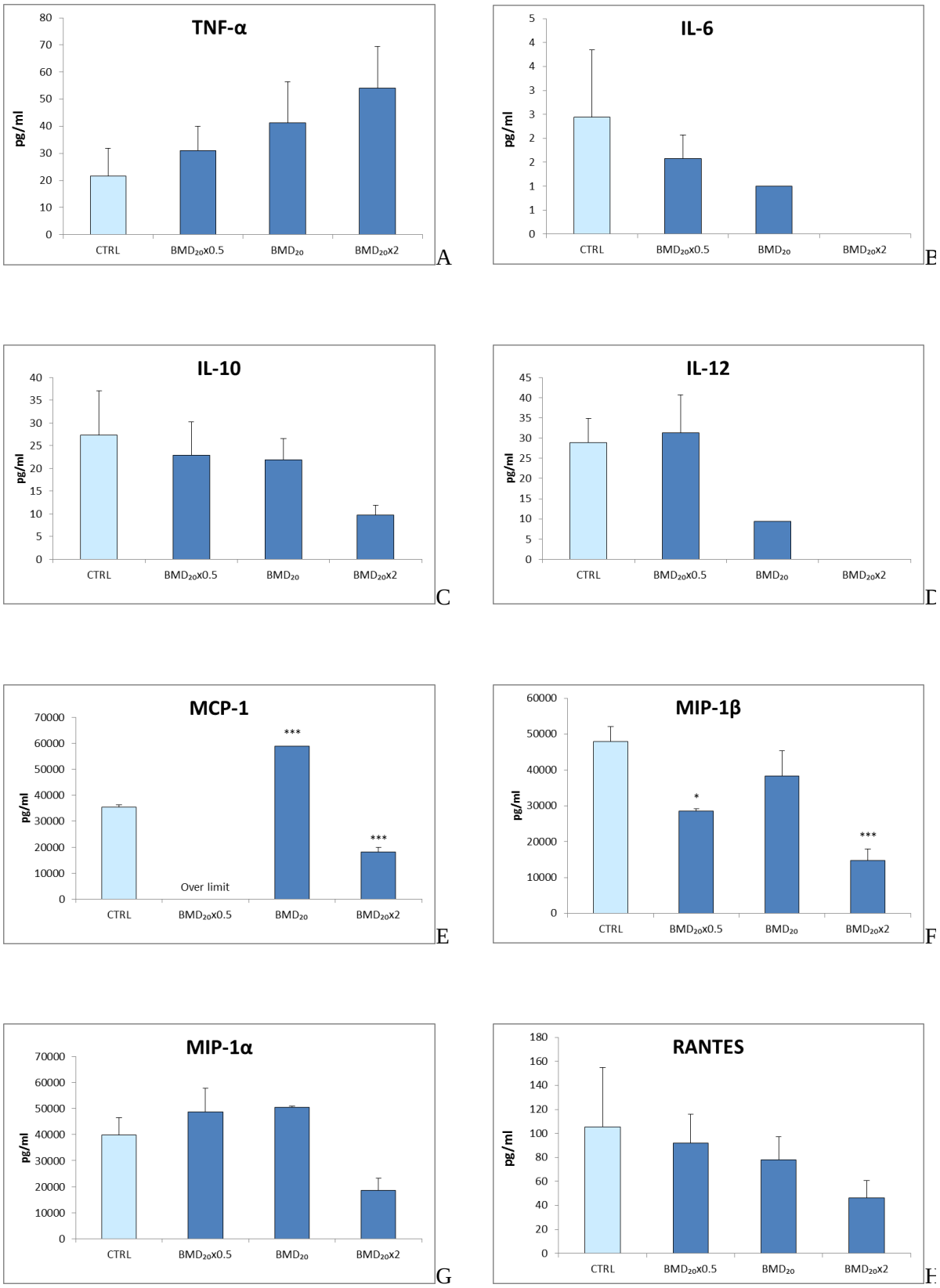


Figure A. 4 Cytokines release by RAW264.7 macrophages after exposure to CuO NMs for 24h.

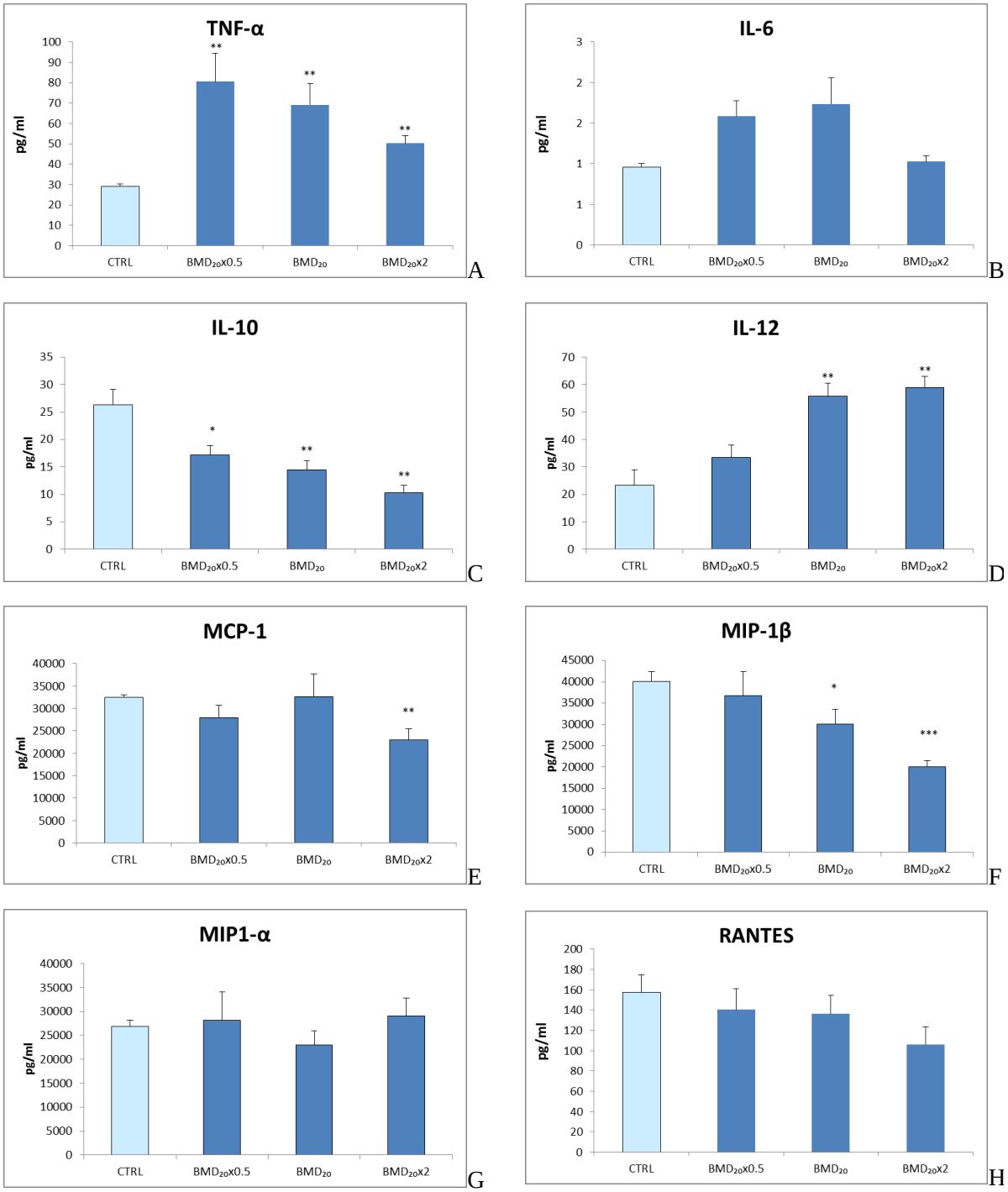


Figure A. 5 Cytokines release by RAW264.7 macrophages after exposure to WCCo NMs for 24h.

Conclusions

The results showed that CuO and WCCo NMs displayed a high toxicity after 24h of exposure in both cell models, macrophages and hepatocytes, CuO being more toxic in comparison with WCCo. The RAW264.7 macrophages showed a higher sensitivity to WCCo NMs compared with the C3A hepatocytes. Further investigations to explain these observations will be necessary including evaluation of NM uptake by these cell lines. The cytotoxicity results were used to guide the experimental design of *in vivo* experiments in FP7-SUN while the cytokine release data was compared with the *in vivo* findings (histopathology, DNA methylation and gene expression) and integrated into the risk assessment process for nanomaterials in a later stage of the project.

Deliverable D 6.3 - Report on molecular, histological, biochemical and epigenetic responses as well as information on biopersistence identified in the STIS with pristine and released/aged NOAA

RIVM - Inhalation exposure

Rats were exposed nose-only to a single exposure concentration, and by varying the exposure time, different dose levels were obtained (C x T protocol). The dose is expressed as 6 hour-concentration equivalents of 0, 0.6, 2.4, 3.3, 6.3 and 13.2 mg/m^3 CuO NPs, with a primary particle size of 10 9.2-14 nm and an MMAD of 1.5 µm.

Twenty-four hours after a 5-day exposure, dose-dependent lung inflammation and cytotoxicity was observed. Histopathological examinations indicated alveolitis, bronchiolitis, vacuolation of the respiratory epithelium and emphysema in the lung starting at 2.4 mg/m^3. After a recovery period of 22 days, limited inflammation was still observed, but only at the highest dose of 13.2 mg/m^3. The olfactory epithelium in the nose degenerated twenty-four hours after exposure to 6.3 and 13.2 mg/m^3, but this was restored after 22 days. No histopathological changes were detected in the brain, olfactory bulb, spleen, kidney and liver.

In conclusion, a 5-day, 6-hour/day exposure equivalent to an aerosol of agglomerated CuO NPs resulted in a dose-dependent toxicity in rats, which almost completely resolved during a 3-week post-exposure period.

The data for all endpoints measured were compared via the BMD calculated via PROAST. This allowed a ranking of the relative sensitivity of each endpoint to the inhaled CuO NP treatment (Figure A.5), with biochemical markers and inflammatory cell number in the bronchoalveolar lavage fluid providing to be the most sensitive indicators for lung toxicity.

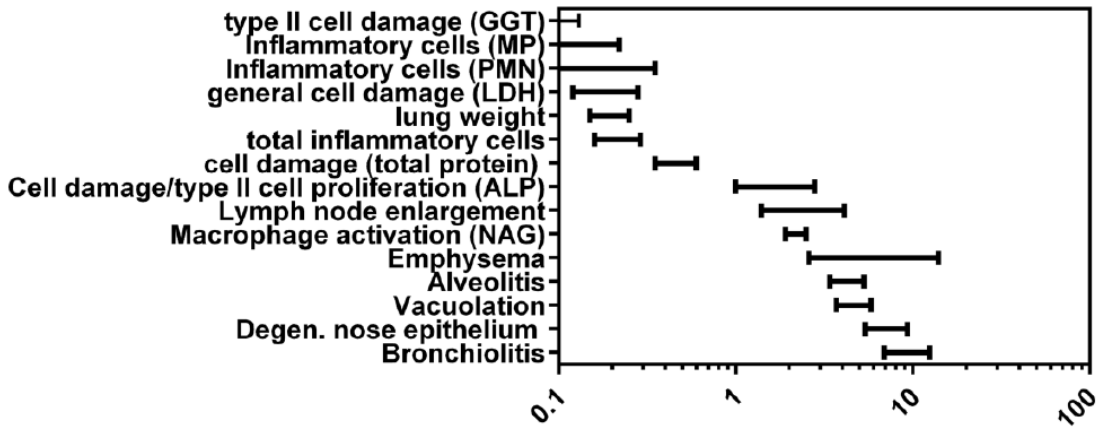


Figure A. 6 Summary of the STIS endpoints. Represented the derived BMDs and confidence interval for all endpoints for which a dose-response was found in the main groups. The dose levels are expressed as 6-hour concentration equivalents in mg/m^3 on a log scale. MP is macrophages, PMN is neutrophils.

RIVM kinetics study

NB. All of the figures and tables referred to in this section are included in the paper by Gosens *et al.* (2016).

The blood, bone marrow, lungs, brain, liver, spleen, kidney, heart, testis and epididymis tissues were freeze-dried and sent ready for ICP-MS analyses for Cu content. The testis and epididymis were very fatty organs, and the freeze dry process did not work very well for those organs. Some organs were dried, but other were still wet and sticky. This gives problems in interpretation of the data expressed as dry organ weights. Therefore, the Cu content of testis and epididymis were not measured.

Organ burdens were assessed 1 day after the last exposure (day 6) as well as after the recovery period (day 28) expressed in µg Cu/g dry tissue (supplementary figure 5, Gosens *et al.*, 2016). The varied concentration equivalents resulted in a different Cu dose in the lung at day 6 (Figure 3A). The measured lung burden was compared with the modelled burden at day 6 using MPPD software by the combined tracheobronchiolar and pulmonary deposition based on the actual aerosol concentration of 11.6 mg/m^3 (highest exposure for the animals dedicated for burden analysis) and the aerosol characteristics as mentioned above (supplementary figure 4, Gosens et al 2016). Using a similar calculation as described in the materials and methods section but including clearance assuming a retention half-time of 60 days, applicable for poorly soluble particles (Oberdorster, 2002), a lung burden of 0.2 mg at day 6 was estimated. The measured lung burden was linear with increasing equivalent concentration (Figure 3B, Gosens et al. 2016). The measured total load for the highest exposed group, 1 day after the final exposure, was 0.085 mg per lung. This was only 43% of the modelled lung burden. Within the 22 day recovery period, Cu was cleared completely from the lung and levels returned to baseline (Figure 3A, Gosens *et al.* 2016). Therefore, the CuO NPs do not follow the kinetics of poorly soluble particles.

To examine the solubility of CuO NPs, dissolution was determined in Gamble's solution (pH 7.4) and artificial lysosomal fluid ALF (pH 4.5) (table 2, Gosens *et al.* 2016). At pH 7.4, around 2% of CuO was present as Cu^{2+} ions. However, at a lower pH, representative of the internal milieu of phagocytizing cells, 60% of CuO NPs mass rapidly dissolved within 1 hour (table 2, Gosens et al 2016). During the dissolution measurements, the hydrodynamic diameter and ζ-potential of the CuO NPs was determined by DLS and ELS, in both Gamble's solution (supplementary table 2, Gosens *et al.* 2016) and ALF (supplementary table 3, Gosens et al. 2016). In Gamble's solution, particle agglomeration was observed, reaching approx. 1 µm, followed by sedimentation, due to the presence of salts, primarily NaCl and $NaHCO_3$. After 24 hours, no particle size distribution could be determined due to a low-intensity signal from the DLS. ζ-potential values suggested the high instability of the dispersions (-5±0.4 mV), confirming agglomeration and sedimentation of CuO NPs. In ALF, CuO NPs dissolved very quickly, and therefore the hydrodynamic particle size distribution could only be determined at time point 0, directly after preparing the suspensions. A bi-modal size distribution was observed at time point 0: 8% of CuO NPs had an average size of 48±8 nm, and 92% reached an average size of 231±51 nm. In this case, ζ-potential values could not be obtained due to the relatively rapid dissolution rate (from seconds to minutes), confirmed also by the intensity values of DLS (supplementary table 3, Gosens *et al.* 2016).

No exposure-related increases above background Cu levels were observed in organs other than lungs, such as liver, blood, bone marrow, brain, heart, kidney and spleen (Figure A.6). A slight decrease in Cu levels was found in the liver after the recovery period. This is most probably related to a non-statistically significant increase in liver weight in the recovery group (data not shown).

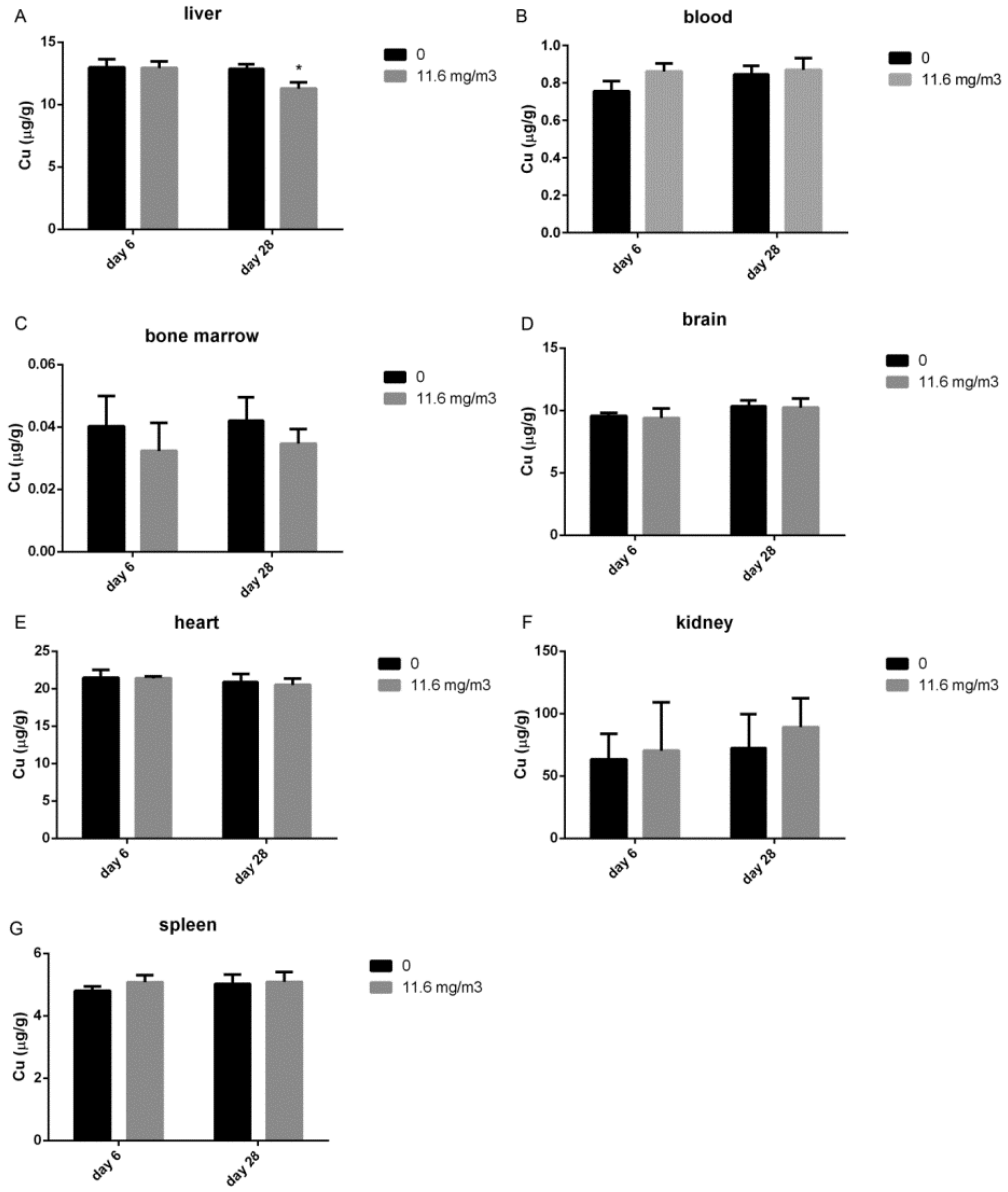


Figure A. 7 Organ burdens. Organ burdens (in µg/g organ dry weight) were assessed 1 day after the last exposure (day 6) and after a recovery period of 22 days (day 28) in control animals and animals exposed to 11.6 mg/m^3 CuO for 6 hours per day, for 5 consecutive days in A) liver, B) blood, C) bone marrow, D) brain, E) heart, F) kidney and G) spleen. The dose levels are expressed as 6-hour concentration equivalents in mg/m^3. $P < 0.05$ compared to control.

HWU Cytokine estimations in lung and liver homogenates

The tissue from this study comprised lung and liver from rats inhaling different airborne mass concentrations of pristine CuO particles. A control group of animals inhaled air only and tissues were obtained at day 6. Each group consisted of five animals, but in some conditions, there were missing samples due to the tissue being used for other purposes.

Approximately 5mm x 5mm pieces of frozen lung or liver tissue obtained from the exposed animals were defrosted and placed in 1.5ml narrow Eppendorf tubes. The homogenisation process was carried out on ice. Two hundred and fifty microliters of lysis buffer (containing 150mM NaCl, 50mM Tris buffer pH 8.0, 1% Triton and 1 Sigma antiprotease tablet) was added to each tube and the tissue finely chopped using surgical scissors. Homogenisation was completed using a VWR VDI 12 hand held homogeniser (setting number 3) for 20 seconds. Samples were then rapidly frozen in liquid N_2 and defrosted at room temperature after which they were sonicated for 20 seconds in a sonic bath. Samples were centrifuged at 4500g for 4 minutes at 4ºC and the supernatant transferred to clean Eppendorf tubes, labelled and stored at -80ºC until required.

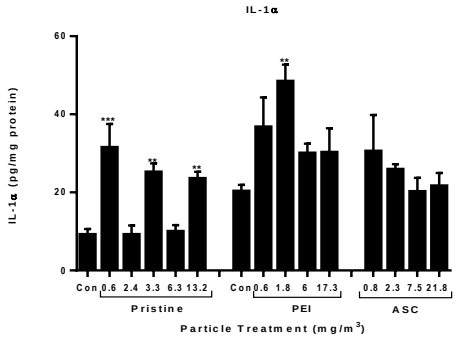
Homogenate samples were diluted 1:20 in PBS and the protein content measured using fluorescamine (1.5mg in 10mls DMSO). A series of BSA standards ranging from 1000µg/ml to 0 µg/ml in PBS was prepared. Twenty-five microliters of standard or diluted homogenate were pipetted into duplicate wells of a 96 well plate and 25µl of stock fluorescamine solution added to each well. The plate was covered with foil and placed on a shaker for 2 minutes, after which the plate was read on a plate reader set to measure at 360nm excitation and 450nm. The protein content of each sample was calculated using a linear regression obtained from the standard curve.

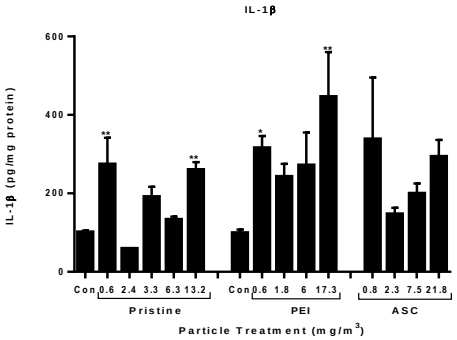
The cytokine measurements were carried out using the Magpix (BioRad) technology. Cytokines of interest (IL-1α, IL-1β, IL-6, IL-12, IL-13, TNF-α and MIP-2) were selected, and a master mix of coated magnetic beads was prepared. The beads were added to duplicate groups of wells on a black 96 well plate, and diluted tissue homogenates added. A series of cytokine standards were included. After incubation, the beads were washed and treated with detection antibody, followed by another series of washes. Finally, PE-labelled streptavidin was added. The fluorescence obtained in each well was measured using a Magpix plate reader, each cytokine of interested was detected in different channels of the, and the concentration was calculated using a four-parameter curve calculated from the standards.

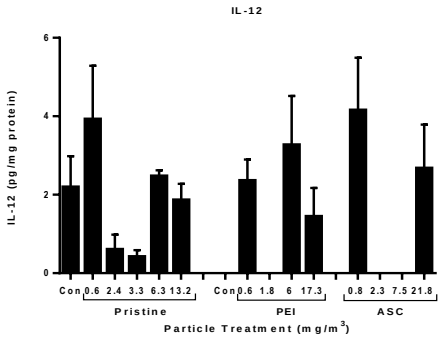
Data from the experiments were analysed using the Minitab statistical package. Analysis of variance using a general linear model and Tukey's post-test was used. Significance was set at 5%.

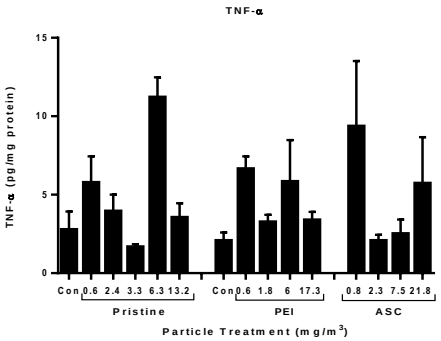
Figure A.8 shows the concentration of each cytokine present in lung and liver tissue. The cytokine concentration has been normalised to the protein content. Note that Figure 6 also contains data for the modified CuO which will be described and discussed in subsequent sections of the report.

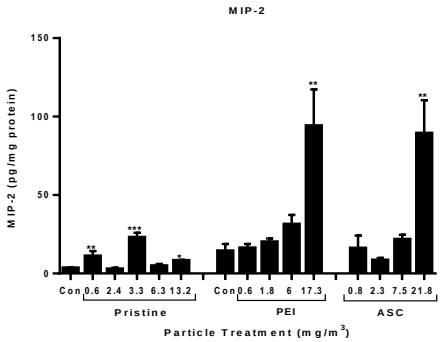
6 Day Lung Homogenates



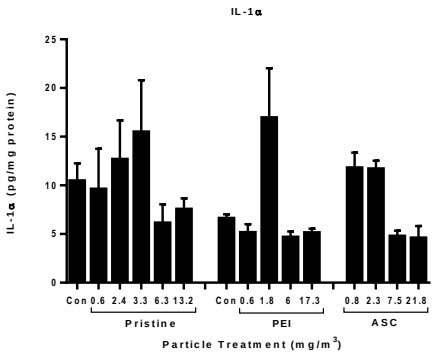


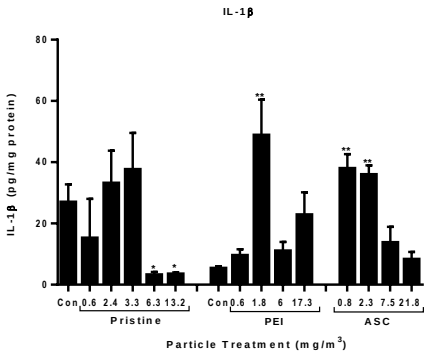


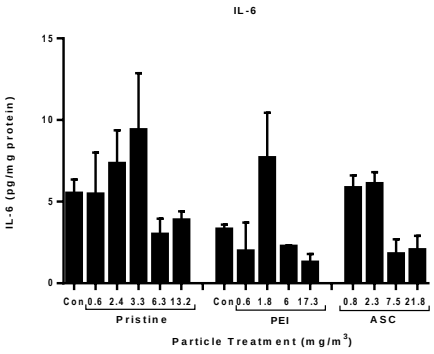


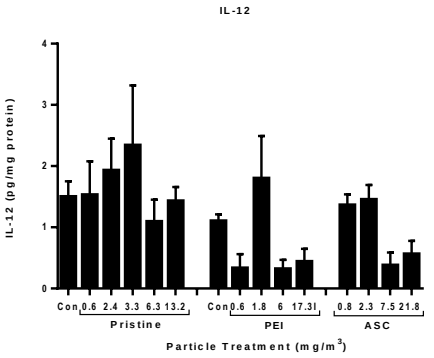


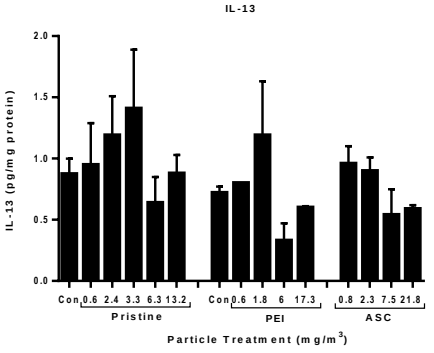
6 Day Liver Homogenates

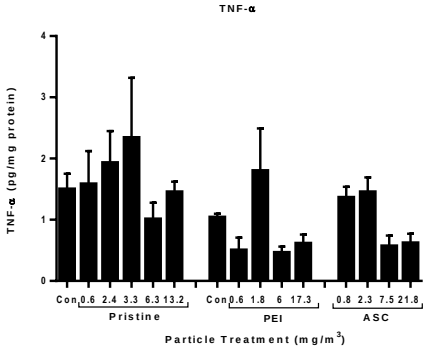












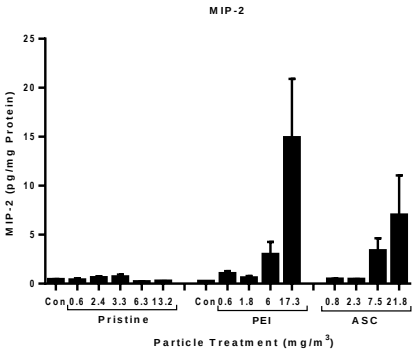


Figure A. 8 Cytokine analysis. Cytokine concentration in 6 day lung and liver homogenates from rats inhaling pristine, PEI and ASC coated CuO particles. Data is normalised to the amount of protein present in each homogenate and represents the mean ±SEM of the number of pg/mg protein from five separate animals.

There was no detectable IL-6 or IL-13 in any of the lung homogenates. For the pristine particles, there was no clear dose effect. IL-1α was significantly increased at the 0.6, 3.3 and 13.2 mg/m^3 doses compared with the control. This pattern was similar to IL-1β concentrations, although there was no significant increase at the 3.3 mg/m^3 dose. Both IL-12 and TNF-α showed a degree of variation and no statistically significant increase was observed at any concentration. In the case of MIP-2, the cytokine level was significantly greater than the control at doses of 0.6, 3.3 and 13.2 mg/m^3.

Inhalation of pristine CuO particles did not cause any significant increase in any of the cytokines investigated in the liver at day 6. However, at 6.3 and 13.2 mg/m^3 particle concentrations, there was a significant reduction in the amount of IL-1β. A similar profile with pristine particles was observed across all of the cytokines investigated, but there were no other significant effects.

Karolinska Institutet and Health Canada epigenetics and transcriptomics

KI performed epigenetic analyses on genomic DNA extracted from the lung samples supplied by RIVM using the AllPrep DNA/RNA Mini kit for animal tissue (Qiagen). Due to the nature of exposure, this organ was prioritised over the liver, for which samples were also received. The potential epigenetic effects of exposure to CuO NPs were addressed by analysing the levels of DNA methylation in the lungs exposed to the doses of 6.3 (low dose, LD) and 13.2 (high dose, HD) mg/m^3 for five days and after the 21-day recovery. For the purpose, a panel of 22 inflammation-related genes was selected for analysis. It should be noted that genome-wide DNA methylation array is not readily available for studies on rat tissues, and, moreover, the approach would be prohibitively expensive. Thus, for our study, the EpiTect Methyl II PCR Array was used, coupled with the Epitect Methyl II DNA restriction kit (Qiagen). The assay consists of a qRT-PCR array for the target genes, following extraction of total DNA, and is based on the hindered transcription of methylated genes. The choice of the panel of genes was based on the high relevance of inflammation occurring as a result of exposure, as explained above. However, the levels of gene methylation were overall low (typically < 1%) for all genes (Figure A.9, A), and highly variable, accounting for no significant differences between any experimental treatment, albeit a trend to reduce the methylation in animals exposed to HD for five days. This could, nonetheless, relate to the increased levels of inflammation observed histopathologically in the lungs of these rats. Individually among the genes that were studied, only Fas-associated death domain (FADD) yielded a significant difference towards the controls, in animals exposed to LD for five days, relatively to controls (Figure A.9).

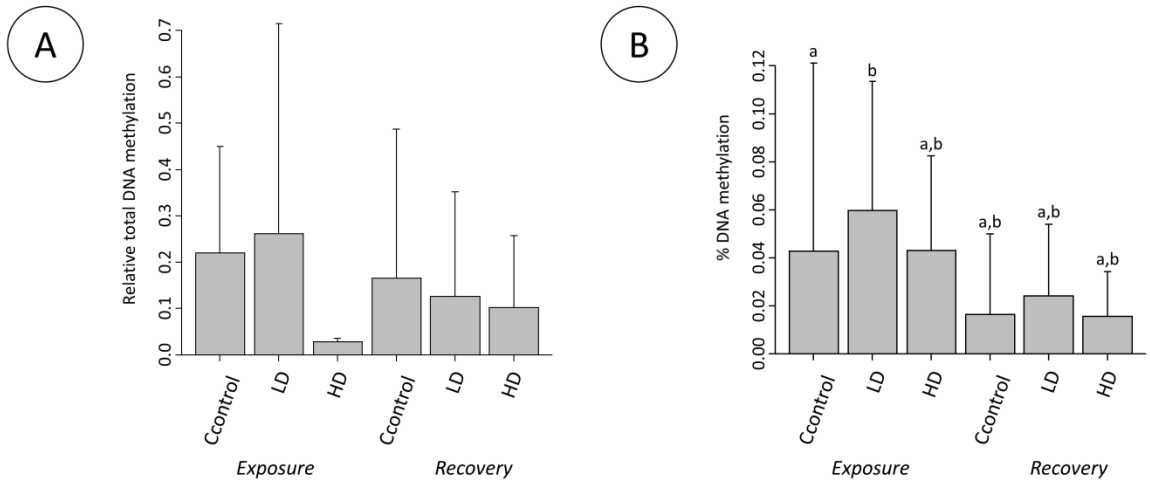


Figure A. *9* DNA methylation assay performed on rat lung tissue. Results expressed as average % of sequence methylation and respective SD. A) Average total methylation among the 22 genes. No significant changes were noted. B) Level of methylation for the FADD gene, for which some degree of statistical significance was found. The letters indicate significant differences (t-test, Bonferroni-corrected p-value < 0.05).

These findings indicated that a relation between epigenetic regulation of gene expression and the reported endpoints related to the inflammatory response in the exposed animals could not be shown, as far as could be ascertained based on the panel of selected inflammation-related genes. Therefore, the corresponding analysis was not conducted on liver samples. Instead, following detailed discussion within WP6 and with the scientific advisory board member, Dr Sabina Halappanavar (Health Canada = HC), the decision was taken to move from epigenetics analyses to gene expression analyses, or *transcriptomics*. Dr Halappanavar thus volunteered to perform microarray-based transcriptomics analyses at no cost, on the samples from WP6. To this end, RIVM provided samples from rat lung tissues to KI, and a protocol was developed at KI for extraction of RNA followed by shipment of samples to HC. Considerable care was taken to ensure that adequate protocols were established to extract RNA samples of high quality for the microarray experiments. The quality of RNAs was assessed through RIN (RNA Integrity Number), which was found to be > 7, as recommended for the purpose. As previous, lung was selected as a priority for being the apical entry organ of toxicants upon inhalation. The microarray study was based on the Agilent Rat Oligonucleotide Platform. Following microarray analysis (at HC), data were transferred to KI and downstream bioinformatics analysis of the data was performed at KI. The results showed that exposure to HD for 5 days yielded the most significant changes in gene expression, accounting for about significantly changed mRNA of about 1,000 genes (considering a minimum fold change ratio of 2, with FDR-corrected $p < 0.05$), the majority of which, up-regulated (Figure A.10). In contrast, samples collected after the recovery period (see above) yielded only < 20 dysregulated genes, thus indicating the return to levels similar to controls, in agreement with the work described above. In-depth bioinformatics including pathway analyses (such as KEGG and Ingenuity Pathway Analysis, IPA), showed that most genes related to inflammation and cell proliferation in exposed animals, again in accordance with the previous results and seemingly in a dose-response manner, albeit reversible after the recovery period.

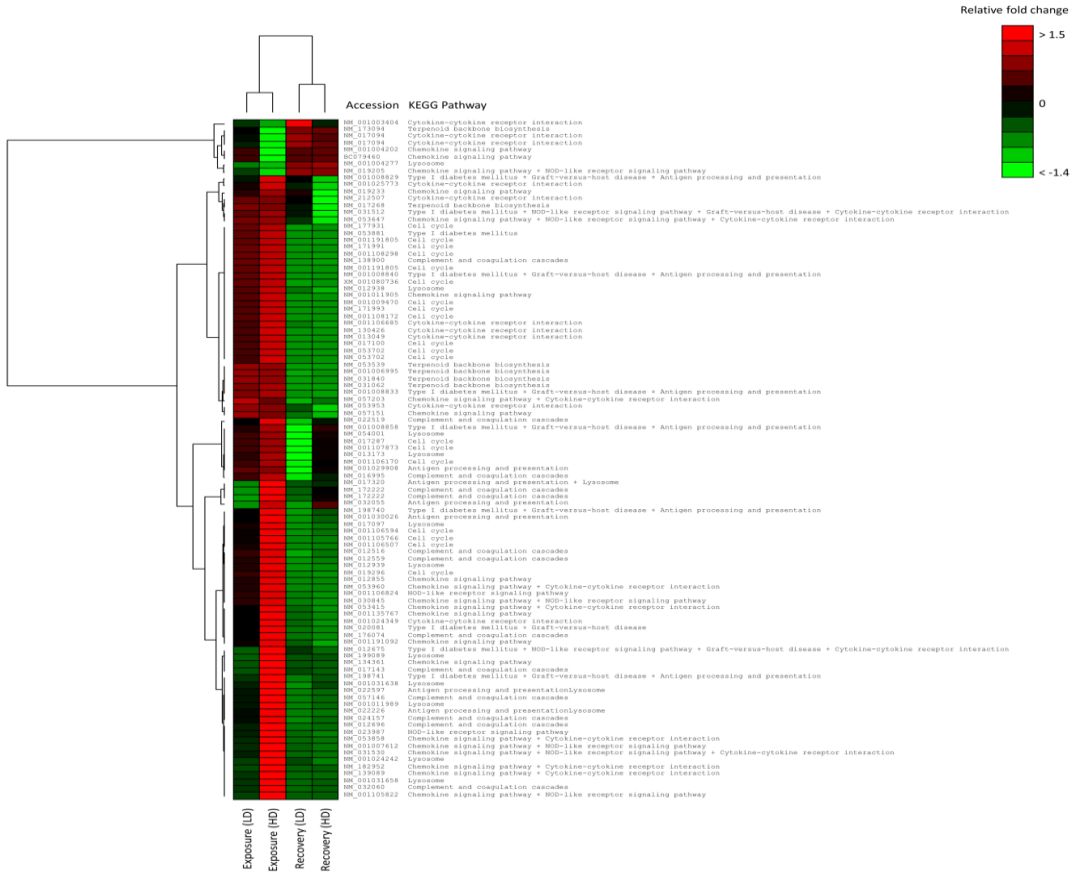


Figure A. 10Heatmap from the microarray experiment performed on lung tissue samples. The map shows the differences in mRNA levels between the experimental conditions. The data presented here refer to ≈100 genes allocated within significantly activated biological pathways according to KEGG pathway analyses (Benjamini-corrected p <.0.05), mostly related to cell proliferation and inflammation

.

Furthermore, detailed pathway analyses were done using the IPA software through a comparative approach between all experimental conditions. The results confirmed the relevance of inflammation and cell proliferation pathways as a response to insult in LD and HD-subjected animals (Figure 8.11). No significantly changed, i.e., activated or deactivated, pathways were found after recovery, indicating a reversal of effects back to the level seen in control animals. In sum, the study provided molecular evidence for the reversibility of effects. In addition, these studies also showed that also the short-term exposure regimen resulted in changes as a consequence of the association between inflammation and hyperplasia in lung bronchoalveolar tissue, which is regarded as a potential pre-neoplastic condition. No evident correlations were found between the epigenetics results and transcriptomics results (data not shown). Overall, the transcriptomics studies have added further insight regarding molecular events following short-term inhalation exposure to CuO NPs.

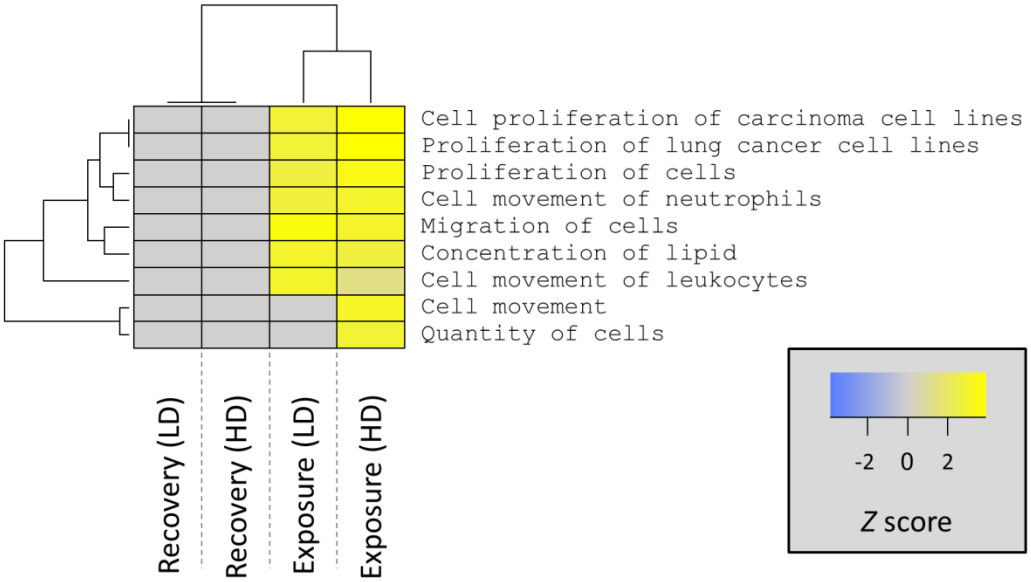


Figure A. 11 Heatmap derived from Ingenuity Pathways (IPA) analysis comparing the four experimental conditions. The data set was contrasted to all available databases, including in vivo and in vitro lung models. The threshold for acceptance of activation/deactivation of pathways was considered to be $p < 0.05$, $Z < -2$ (deactivation) and $Z > 2$ (activation).

The abovementioned findings from the microarray experiments were then verified immunohistochemically by KI using fluorescent-labelled secondary antibodies (IHC-IF) from paraffin-embedded samples (supplied by RIVM) using several probes, such as the anti-Ki67 antibody for the localization of proliferating cells, using fluorescence microscopy (IF) (Figure A.12). Both the microarray experiments and IHC-IF analysis state inflammation and alveolar epithelial cell proliferation as the main changes caused by exposure, albeit reversible after removal of a challenge. These effects occurred in an obvious dose-response manner and confirmed that exposure to CuO NPs by inhalation yield both epithelial cell proliferation and inflammation. A manuscript is currently being finalized by KI in collaboration with HC and RIVM.

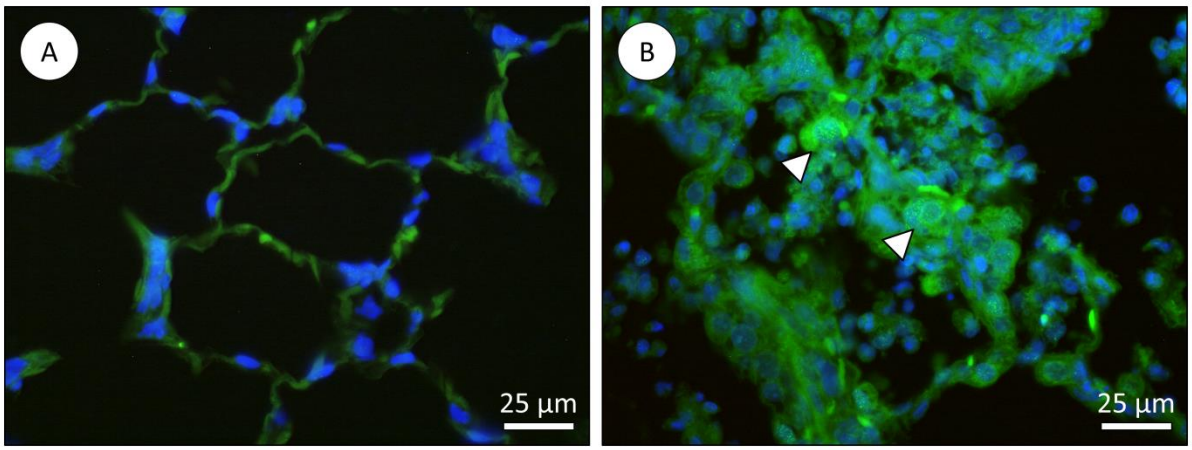


Figure A. 12 Micrographs from the IHC-IF analyses in rat lungs, using the anti-Ki67 antibody as a probe for cell proliferation in rat alveolar epithelia. A) Control rats. B) Rats exposed to HD for 5 consecutive days. Note alveolar epithelial hyperplasia, infiltration of immune cells and upregulation of the Ki67 protein (arrowheads), stained green/yellow, confirming cell proliferation. Counterstain: DAPI (blue).

Second STIS - Safety by Design Polyethylenimine and Ascorbate Modified CuO

The original plan had been to use fragmented particles (FP) from a polymer composite including CuO (WP 5). However this was unsuccessful due to the soft nature of the composite. Since WP7 was also making modified CuO in order to improve their safety by design, the modified CuO were considered for the inhalation study.

Initially, discussions were held with WP7 partners to consider the range of surface modifications under consideration. The toxicology partners of WP6 suggested that ascorbate (ASC) might we worth considering as a novel surface modification. Ascorbate is negatively charged and so should improve the suspension stability, but in addition, ascorbate is an antioxidant found naturally in the body, including in lung lining fluid. The natural occurrence of ascorbate and its antioxidant properties should help to mitigate any NP (or even Cu^{2+}) induced toxicity. The partners of WP7 utilised this idea to successfully generate the ascorbate coating. In addition, they provided citrate, polyethylenimine coating (PEI) and polyvinylpyrrolidone PVP coated CuO NP (D7.1).

KI and HWU *in vitro* studies to guide the *in vivo* study design

As a step to prepare the STIS bioassays with pristine CuO NPs, this ENM was tested *in vitro* using the RAW264.7 macrophage-like cell line (KI) and C3A hepatocytes (HWU), as described in Deliverable 6.1. This was followed by a series of assays with the same cell lines, also conducted at KI and at HWU to test the cytotoxicity of the different surface-modified CuO

NPs, namely with the anionic sodium citrate (CIT), sodium ascorbate (ASC); the neutral polyvinylpyrrolidone (PVP) and the cationic polyethylenimine (PEI). The results were evaluated using the benchmark dose approach using the PROAST software package for R and data are reported in Table A.2 (below). While differences could be discerned for the different modified CuO NPs, the cytotoxicity was not entirely resolved, as the modified (capped) NPs retained their toxicity (the particles were not "non-cytotoxic"). Nevertheless, ASC-modified NPs were the least cytotoxic for both cell lines (see BMD_{20} values in Table A.2). Cytotoxicity of modified NPs to RAW264.7 macrophages was ranked as PEI-capped > uncapped NPs > PVP-capped > CIT-capped > ASC-capped. The findings were similar to the results for C3A hepatocytes, except that PVP-coated NPs were found to be the most cytotoxic.

Furthermore, the effects of capping agents on particle dissolution were addressed by KI using RAW264.7 macrophages, suggesting that only marginal differences occurred between the distinct synthesised materials and only for a brief period, since after 24 h the dissolution rates of NPs in DMEM were similar (≈ 66%). Overall, the cytotoxicity of modified CuO NPs tested with this *in vitro* model seems to result from the interaction between NP and capping agent toxicity and neither by Cu burden *per se* or NP internalization, which was verified by ICP-MS and transmission electron microscopy. Altogether, this information was used to guide the subsequent *in vivo* assays, as previously decided by the teams involved. A manuscript is currently being produced with the *in vitro* data on surface-modified CuO NPs, in collaboration with partners from WP7, who manufactured and supplied the tested NPs.

Table A. 2 Benchmark dose estimates (BMD_{20}), in µg/ml, and respective 95% upper and lower bound obtained from in vitro assays with macrophages (RAW267.4) and hepatocytes (C3A).

Capping agent	**RAW267.4**	**C3A**
Uncapped	2.74 (2.14 - 3.42)	18.7 (16.81 - 20.64)
Citrate	10.60 (9.03 - 12.24)	25.7 (23.07 - 28.58)
Ascorbate	11.70 (9.23 - 13.48)	64.8 (59.02 - 70.81)
PVP	5.06 (4.34 - 5.81)	16.6 (14.77 - 18.55)
PEI	1.65 (1.12 - 2.33)	20.4 (18.43 - 22.36)

Thus, on the basis of the *in vitro* results reported above, CuO NP modified with a PEI (positively) charged and ASC treatment (resulting in a negative charge) were tested in the subsequent STIS. This also allowed the relative toxicity of positive and negatively charged CuO NPs compared to the pristine material to be addressed.

The hypothesis was that the positively charged particles would render the particles more toxic compared to the unmodified particles, while the negative charged coating will protect against toxic effects. A positively charged particle could be more easily recognized by the immune

system (macrophages), while the ascorbate coating could reduce the Cu^{2+} ions (that are linked to the toxicity) to Cu0, or the ascorbate forms a complex with the Cu^{2+} ions or both.

The test item-related toxicological relevant morphologic alterations following the administration of CuO nanoparticles (positively coated with PEI or negatively coated with ASC) for 5 days to rats were present mainly in the lung and its draining lymph node, the mediastinal lymph node. Findings consisted of interstitial/alveolar inflammation and hypertrophy/hyperplasia of bronchioles/alveoli with accompanying alveolar (cellular) debris in the lung and paracortical histiocytosis in the mediastinal lymph node. Although all treatment groups were affected, the findings starting at a dose of 1.8 mg/kg^3 CuO-PEI and 2.3 mg/ kg^3 CuO-ASC were considered to be adverse, based on the nature and severity of the findings. After 21 days, recovery was ongoing since the number of alveolar macrophages was still slightly increased at all doses of both CuO-PEI and CuO-ASC, and two males treated at 17.3 mg/kg^3 still showed minimal bronchiolar hypertrophy/hyperplasia.

All data have been collected and entered in the data transfer sheets (Table A.3). BMD analyses are in progress. Initial considerations of the data suggest that like the pristine CuO NP, both of the modified CuO NP induce a significant inflammation at day 6. For the PEI coated NPs, a residue of inflammatory cell accumulation remains at day 28; again this is comparable to the pristine CuO NP. For the ASC modified CuO NPs, there appears to be no inflammatory cell accumulation at day 28 for any exposure concentration, suggesting that the ASC may have provided some protection preventing the inflammatory effects persisting to day 28. This needs to be verified via statistical analysis of the data.

Table A. 3 The result of cell differentials in BALF showing dose-dependent effects.

DAY 6

				abs. aantal (x 10^6/ml pellet)					
	n		celconc. (10^6/ml pellet)	Macro-fagen	Macrof. meerk.	Neutrof. granuloc.	Eosinof. granuloc.	Lymfoc.	Monoc.
Groep 1, clean air 0 mg/m3, 3h	5	gemid.	0,488	0,458	0,003	0,021	0,001	0,005	0,000
		sd	0,191	0,174	0,002	0,018	0,002	0,004	0,000
Groep 2, CuO PEI 0.6 mg/m3, 7 min.	4/3	gemid.	0,805	0,464	0,008	0,127	0,000	0,004	0,000
		sd	0,158	0,318	0,008	0,102	0,001	0,003	0,000
Groep 3, CuO PEI 1.7 mg/m3, 20 min.	5	gemid.	2,848	0,337	0,022	2,442	0,008	0,037	0,002
		sd	0,883	0,277	0,023	0,944	0,005	0,060	0,005
Groep 4, CuO PEI 5.0 mg/m3, 1h.	5	gemid.	6,260	1,095	0,025	4,742	0,009	0,386	0,003
		sd	0,755	0,403	0,034	0,811	0,008	0,195	0,007
Groep 5, CuO PEI 15 mg/m3, 3h.	5	gemid.	12,481	1,867	0,000	10,233	0,002	0,378	0,000
		sd	5,342	1,056	0,000	4,643	0,004	0,287	0,000
Groep 6, CuO ACS 0.77 mg/m3, 7 min.	5	gemid.	0,941	0,453	0,005	0,456	0,006	0,020	0,000
		sd	0,302	0,404	0,005	0,184	0,003	0,026	0,000
Groep 7, CuO ACS 2.2 mg/m3, 20 min.	5/4	gemid.	3,145	0,662	0,026	2,122	0,008	0,149	0,051
		sd	0,983	0,122	0,015	0,888	0,012	0,123	0,096
Groep 8, CuO ACS 6.7 mg/m3, 1 h.	4	gemid.	6,786	1,361	0,014	4,847	0,019	0,472	0,073
		sd	2,160	0,650	0,010	1,409	0,018	0,189	0,046
Groep 9, CuO ACS 20 mg/m3, 3 h.	5	gemid.	14,814	2,444	0,021	11,392	0,017	0,816	0,123
		sd	3,901	1,071	0,036	3,702	0,024	0,311	0,119

DAY 27

	n		celconc. (10^6/ml pellet)	Macro-fagen	abs. aantal (x 10^6/ml pellet) Macrof. meerk.	Neutrof. granuloc.	Eosinof. granuloc.	Lymfoc.	Monoc.
Groep 1, clean air 0 mg/m3, 3h	5	**gemid.**	**0,508**	**0,439**	**0,008**	**0,041**	**0,001**	**0,020**	**0,000**
		sd	0,093	0,137	0,006	0,048	0,001	0,032	0,000
Groep 2, CuO PEI 0.6 mg/m3, 7 min.	4	**gemid.**	**0,413**	**0,352**	**0,011**	**0,043**	**0,000**	**0,007**	**0,000**
		sd	0,077	0,105	0,001	0,028	0,001	0,004	0,000
Groep 3, CuO PEI 1.7 mg/m3, 20 min.	5	**gemid.**	**0,523**	**0,455**	**0,004**	**0,051**	**0,000**	**0,013**	**0,000**
		sd	0,140	0,113	0,004	0,051	0,000	0,008	0,000
Groep 4, CuO PEI 5.0 mg/m3, 1h.	5	**gemid.**	**0,694**	**0,646**	**0,016**	**0,022**	**0,000**	**0,010**	**0,000**
		sd	0,274	0,286	0,009	0,028	0,000	0,006	0,000
Groep 5, CuO PEI 15 mg/m3, 3h.	5	**gemid.**	**0,463**	**0,388**	**0,009**	**0,056**	**0,000**	**0,011**	**0,000**
		sd	0,187	0,182	0,006	0,100	0,000	0,013	0,000
Groep 6, CuO ACS 0.77 mg/m3, 7 min.	5	**gemid.**	**0,758**	**0,670**	**0,011**	**0,062**	**0,001**	**0,014**	**0,000**
		sd	0,130	0,116	0,006	0,052	0,001	0,011	0,000
Groep 7, CuO ACS 2.2 mg/m3, 20 min.	4	**gemid.**	**0,604**	**0,568**	**0,014**	**0,017**	**0,000**	**0,005**	**0,000**
		sd	0,237	0,235	0,008	0,012	0,000	0,004	0,000
Groep 8, CuO ACS 6.7 mg/m3, 1 h.	5	**gemid.**	**0,525**	**0,497**	**0,021**	**0,004**	**0,000**	**0,004**	**0,000**
		sd	0,139	0,136	0,009	0,003	0,000	0,002	0,000
Groep 9, CuO ACS 20 mg/m3, 3 h.	5	**gemid.**	**0,487**	**0,458**	**0,016**	**0,005**	**0,000**	**0,008**	**0,000**
		sd	0,158	0,158	0,009	0,003	0,000	0,006	0,000

HWU Cytokine estimations in lung and liver homogenates

Again, there was no detectable IL-6 or IL-13 in any of the lung homogenates. With both modified particle types, there was a clearer effect of dose of particles compared with the effects observed with the pristine particles. The PEI coated NP produced an increase in IL-1α at the 1.8 mg/m^3 dose compared with the control and a significant increase in IL-1β at the 0.6 and 17.3 mg/m^3 doses. Similarly, there was an increase in MIP-2 at the highest dose of 17.3 mg/m^3. No significant increase in IL-12 or TNF-α was observed after PEI modified CuO particle inhalation at any concentration. The ASC modified CuO NP did not cause any increased cytokine release in the lung except for MIP-2, which was significantly greater than the control at the dose of 21.8 mg/m^3. Therefore, in the lung, the ASC modification appeared to dampen the pro-inflammatory cytokine signalling compared to the pristine and PEI modifications.

Inhalation of pristine CuO particles did not cause any significant increase in any of the cytokines investigated in liver tissue. With the modified particles, there was only a significant increase in IL-1β for the PEI coating at 1.8 mg/m^3 and for ASC at 0.8 and 2.3 mg/m^3.

KI – DNA methylation and microarray analysis

As stated above, the findings from the epigenetics study within the previous STIS experiment revealed little correlation between DNA methylation and inflammation or other relevant endpoints. Therefore, it has been decided to replace these analyses with a microarray-based

"omics" study. As before, RIVM will supply the samples, KI will extract RNA following established protocols and will supply the samples to HC for microarray analysis (at no cost). The results of the microarray study are anticipated in June 2016 and will be followed by detailed bioinformatics analysis, performed at KI, and follow-up studies will be considered as needed.

Deliverable D 6.4 - Report on molecular, histological, biochemical and epigenetic responses as well as information on biopersistence identified in the STOS with pristine and released/aged NOAA

Similar to the short-term inhalation study (STIS) in Deliverable 6.3 an oral study was developed to investigate possible toxic effects after short-term oral exposure. Based on the gap analysis as performed in WP1 of the SUN project CuO was the first nanomaterial (NM) investigated in this short-term oral study (STOS). CuO is used as a wood preservative, and so oral exposure is possible by hand to mouth contact. For a second NM, one of the industrial partners suggested that $CuCO_3$ nanomaterials would be a useful comparison with CuO as it is also widely used in wood preservatives. Therefore, as a second NM $CuCO_3$ was selected to perform a STOS for comparison. Both for CuO and $CuCO_3$ oral exposure is deemed to be an important potential route of exposure.

The aim of these studies was to evaluate whether 5 days oral exposure could be indicative for (sub)acute toxicity. In addition, the aim was to evaluate whether already after 5 days of exposure target organs can be identified for further in-depth study in follow-up repeated dose toxicity studies.

Short-Term Oral Study (STOS)

Experimental design - Male rats (RjHan:WI, bred Specific Pathogen Free, barrier maintained during the experiment) of 8-9 weeks old were obtained from Janvier Labs (Le Genest-Saint-Isle, Saint Berthevin , France). The animals were treated on five consecutive days (days 1 - 5), and an autopsy was performed 24 hours after the last oral administration (day 6). In addition, a recovery period of 3 weeks was included in the experiments to evaluate recuperation or possible persistence of the nanomaterials in the body and ongoing pathology. An autopsy of the recovery groups was performed after a three weeks recovery period at day 26.

For CuO initial doses were selected based on literature information (see below). A pilot study was performed with a high 64 mg/kg dose (day 1-5 daily). For $CuCO_3$ a pilot study was performed to determine the starting dose in the STOS. One animal was treated orally with a single dose of $CuCO_3$. If no toxicity was observed the dose was increased in the next animal until a toxic response was noted (OECD 425, 2008, OECD, Paris, France). When a single animal showed a toxic response additionally, 4 animals were administered the same dose. The highest dose chosen for the CuO was 512 mg/kg and for $CuCO_3$ 128 mg/kg.

Exposure - CuO NM was orally administered by gavage using the following exposure doses: vehicle control, 1, 2, 4, 8, 16, 32, mg/kg body weight (b.w.) and a pilot study with 64 mg/kg b.w. The doses were chosen based on information in the literature of soluble non nano $CuSO_4$ which indicated a no observed adverse effect level (NOAEL) of 16.3 mg/kg (Hébert C.D. 1993). The dose was administered as 0.1 ml per 20 g (1 ml per 200 g). In an additional study, one group of animals (n=4) was treated with a high dose of 512 mg/kg b.w. Information on copper published by the EU-RAR is presented on the ECHA website (http://echa.europa.eu/copper-voluntary-risk-assessment-reports).

CuO and $CuCO_3$ nanoparticle dispersions were prepared starting with CuO powder and $CuCO_3$ liquid. Milli-Q water was used for further dispersion and/or dilution. After dispersion, the CuO and $CuCO_3$ nanoparticle dispersions were sonicated for 16 minutes in a water bath (Elmasonic S100) at room temperature to optimize dispersion quality.

Before each administration, the nanoparticle dispersion was evaluated by CPS Disc Centrifuge™ (CPS Instruments Europe, Oosterhout, The Netherlands) to determine nanoparticle size and size distribution. Both the CuO and the $CuCO_3$ dispersions remained stable up to 24 and 72 hours, respectively, after preparation of the dispersion.

Pathology - Potential toxicological effects of 5 days oral administration of CuO and $CuCO_3$ was evaluated 24 hours after the last administration (indicated with day 6 in the tables/figures). In addition, a recovery period of three weeks was included to evaluate possible recovery from toxic insults present after the treatment at day 6 (indicated as day 26). Animals were killed by exsanguination from the abdominal aorta during anaesthesia by isoflurane (3.5% in oxygen) inhalation. General post-mortem examinations were performed on all animals. At the time of necropsy, the following tissues and organs were collected: Heart, Lung, Thymus, Liver, Spleen, Kidney, Testis (unilateral), Brain, Mesenteric lymph node, Adrenal glands, Pancreas, Prostate, Seminal vesicles, Epididymides, Thyroid gland, Skeletal muscle -quadriceps, Stomach, Duodenum, Jejunum, Ileum, Peyer's patches, Cecum, colon, rectum, Skin, Popliteal lymph node, femur with bone marrow, Bone with bone marrow, and Peripheral Nerve.

Microscopic examination of routinely prepared hematoxylin-eosin stained paraffin sections was performed on heart, lungs, thymus, stomach, duodenum, jejunum, ileum, Peyer's patches, cecum, colon, rectum, liver, spleen, mesenteric lymph nodes, kidneys, adrenal glands, brain, testis (unilateral), femur with bone marrow of animals given in the study design table above. In addition, macroscopic findings were prepared and examined. Investigated were all control groups and 32, 64 and 512 mg/kg CuO treated animals. For $CuCO_3$ treated animals the following doses were evaluated: controls and 32, 128 mg/kg $CuCO_3$. The animal data and macroscopic lung findings were manually entered into the computer system PathData®. Stained histologic sections were examined by light microscopy, and the microscopic findings were recorded using on-line input under pathology number 41532 JOL.

Severity grades were assigned to non-neoplastic histopathologic diagnoses, as presented below. Severity grades were assigned based on the severity of alterations in the examined histologic sections and may not reflect the overall severity of the pathologic process in the entire tissue, organ, or animal. Histopathological changes were described according to distribution, severity and morphological character. Severity scores for histopathology were assigned as follows:

Present Finding present, grading not scored.

Grade 1 Minimal/very few/very small.

Grade 2 Slight/few/small.

Grade 3 Moderate/moderate number/moderate size.

Grade 4 Marked/many/large.

Grade 5 Massive/extensive number/extensive size.

N.A.D. No Abnormality Detected

Haematology - Blood was collected in EDTA-coated tubes. Haematological parameters included white blood cell (WBC) count, red blood cell (RBC) count, haemoglobin (Hb), haematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCHC), and platelet (PLT) count. All haematology parameters in the blood samples were determined in an Advia 120 Hematology Analyzer (Siemens Health Care, Germany) according to the manufacturer's instructions. In addition, blood smears were prepared for visual evaluation depending on the results obtained with the haematology analyser.

Bone marrow - Cells were collected by flushing 4 ml Impuls Cytometer Fluid through the left femur. The concentration of nucleated cells was determined in a Coulter Counter.

Clinical chemistry - After collection of blood serum and storage at -20^{o} C the following parameters were determined: albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), amylase (AMY), creatinin kinase (CK), lactate dehydrogenase (LDH), sodium (Na), potassium (K), glucose (GLU), urea, creatinin (CRE), cholesterol (CHOL) and total protein (TP). All clinical chemistry parameters in the serum samples were determined in an automatic analyser Unicel DxC 800 Synchron Clinical Systems (Beckman Coulter Nederland B.V., Woerden, The Netherlands).

Cytokine protein content - Cytokine protein content was determined in various tissues from animals dosed orally with CuO or $CuCO_3$. Tissues from 5-day dosed animals previously snap frozen in liquid nitrogen and stored at -200^{o}C were defrosted prior to extraction. The tissue was homogenised in an extraction buffer (as previously described for the inhalation study) and the protein content estimated as before (fluorescamine assay). The cytokine concentration of the tissue extracts was determined as previously described using a Biorad Magpix system. Treatments consisted of CuO at doses of 16 and 32mg/kg, and $CuCO_3$ at doses of 8 to 128mg/kg. The tissues examined included stomach, ileum, duodenum, colon and liver. The panel of cytokines consisted of IL-1α, IL-1β, IL-6, IL-12, IL-13 and TNF-α. Animals were sacrificed at day 6 post dosing the animals during days 1-5 (i.e. 24 hours after the last oral administration).

GSH in liver tissue from animals dosed orally with CuO or $CuCO_3$ - Liver tissue from animals dosed as described above were homogenised as previously described for the inhalation study. The doses examined for the oral treatments included 64 and 128mg/kg $CuCO_3$. The CuO exposures consisted of doses at 16 and 32mg/kg but included a dose of 512mg/kg which was a dose included as a single high dose investigation. The animals from this treatment were sacrificed at day 6 after treatment for 5 consecutive days (days 1-5).

Results: CuO - General toxicity

Assuming that the CuO would be dissolved in the acid environment of the stomach, a starting dose was selected based on the literature for dissolved $CuSO_4$. The no observed adverse effect level (NOAEL) for $CuSO_4$ was reported to be 16.3 mg/kg (RAR-ECHA). A pilot study was conducted with 2 animals with a dose of 64 mg/kg. As this dose was well tolerated a dose response study was conducted with the highest dose of 32 mg/kg.

During the study, no signs of toxicity were noted, and there were no premature deaths in the study. There were no test item related findings at the macroscopic observation level at autopsy. For the extra group of animals treated with 512 mg/kg some indications for toxicity were observed. One animal showed some weight loss after two days of treatment and diarrhoea after three administrations. After four administrations two animals showed diarrhoea.

Between the start of the exposure and autopsy, no loss in body weight was observed for CuO treated animals. For the doses evaluated up to 32 mg/kg no effect on body or organ weights was noted either at day 6 or day 26 (Table A.4 and 5).

Table 1, A. Body and organ weight in male rats after 5 days consecutive oral administration of CuO. At day 6 (24 hours after the last administration).

Table A. 4. Body and organ weight in male rats after 5 days consecutive oral administration of CuO. At day 6 (24 hours after the last administration).

CuO								Pilot Study	Extra Study
Dose	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg	512 mg/kg
Body weight (g)	275 ± 8	275 ± 25	292 ±9	310 ± 46	288 ±8	290 ± 7	280 ± 23	288 ± 4	334 ± 43
Lung	1.30 ± 0.12	1.33 ± 0.10	1.37 ± 0.09	1.53 ± 0.09 (3)	1.53 ± 0.08	1.39 ± 0.08	1.35 ± 0.10	1.29 (1)	1.48 ± 0.09
Heart	0.881 ± 0.02	0.938 ± 0.06	0.957 ± 0.02	0.934 ± 0.06	0.945 ± 0.02	0.976 ± 0.03	0.931 ± 0.06	0.934 ± 0.08	1.097 ± 0.16
Liver	10.67 ± 0.52	11.00 ± 0.47 (3)	11.19 ± 0.62	11.01 ± 0.63	11.22 ± 0.39	10.52 ±0.80	10.89 ± 1.14	11.01 ± 0.19	12.06 ± 2.21
Kidney (L+R)	2.18 ± 0.08	2.28 ± 0.14 (3)	2.31 ± 0.10	2.22 ± 0.18	2.33 ± 0.06	2.33 ± 0.09	2.14 ± 0.26	2.18 ± 0.08	2.25 ± 0.25
Spleen	0.50 ± 0.04	0.50 ± 0.04	0.53 ± 0.03	0.52 ± 0.04	0.53 ±0.03	0.54 ± 0.03	0.54 ± 0.04	0.51 ± 0.06	0.855 ± 0.22
Thymus	0.503 ± 0.04	0.537 ± 0.09	0.552 ± 0.04	0.591 ± 0.02	0.544 ± 0.04 (3)	0.530 ±0.07	0.527 ± 0.06	0.478 ± 0.03	0.526 ± 0.21
Mesenteric Lymph Node	0.126 ± 0.01	0.157 ± 0.05	0.161 ± 0.03	0.154 ± 0.02	0.144 ± 0.02	0.164 ± 0.03	0.155 ± 0.02	0.125 ± 0.02	0.263 ± 0.07
Testes (L+R)	2.66 ± 0.18	2.50 ±0.64	2.83 ± 0.11	2.93 ± 0.10	2.71 ± 0.22	2.80 ± 0.11	2.78 ± 0.14	2.79 ± 0.06	3.31 ± 0.30
Brain	2.00 ± 0.03	2.03 ± 0.01	20.6 ± 0.02	2.02 ± 0.03	2.04 ±0.04	2.07 ± 0.04(3)	20.3 ± 0.06	2.06 (1)	2.11 ± 0.03
Adrenals	0.032 ± 0.001	0.034 ± 0.005	0.037 ± 0.003	0.037 ± 0.002	0.039 ± 0.004 (2)	0.037 ± 0.003	0.039 ± 0.003	-	0.058 ± 0.005

Table A. 5 Body and organ weights in male rats after 5 days consecutive oral administrations of CuO. At day 26 (21 days after the last administration).

Dose	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg
Body weight (g)	343 ± 7	332 ± 9	351 ±14	318 ± 56	345 ± 12	329 ± 41	341 ± 14
Lung	1.47 ± 0.08	1.54 ± 0.20	1.51 ± 0.07	1.50 ± 0.16 (3)	1.53 ± 0.10	1.41 ± 0.18	1.52 ± 0.17
Heart	1.011 ± 0.04	0.987 ± 0.08	1.037 ± 0.08	0.992 ± 0.07	1.012 ± 0.08	1.052 ± 0.04	1.019 ± 0.06
Liver	12.36 ± 0.27	11.67 ±0.72	12.40 ± 0.70	11.03 ± 1.99	12.80 ± 1.12	11.18 ± 2.24	11.92 ± 0.42
Kidney (L+R)	2.61 ±0.06	2.46 ± 0.25	2.61 ± 0.12	2.38 ± 0.42	2.38 ± 0.32	2.45 ± 0.52	2.78 ± 0.39
Spleen	0.582 ± 0.03	0.521 ±0.04	0.582 ± 0.03	0.436 ± 0.17	0.595 ± 0.03	0.540 ± 0.05	0.599 ± 0.11
Thymus	0.483 ± 0.07	0.407 ± 0.04	0.486 ± 0.04	0.466 ± 0.06	0.485 ± 0.03	0.433 ± 0.13	0.493 ± 0.06
Mesenteric Lymph Node	0.189 ± 0.04	0.173 ± 0.01	0.200 ± 0.01	0.169 ± 0.01	0.161 ± 0.02	0.159 ± 0.02	0.204 ± 0.001
Testes (L+R)	2.97 ± 0.09	2.86 ± 0.06	3.10 ± 0.21	2.77 ± 0.41	2.89 ± 0.24	2.91 ± 0.37	2.98 ±0.07
Brain	2.14 ± 0.02	2.09 ± 0.04	2.15 ± 0.02	2.08 ± 0.09	2.10 ± 0.03	2.08 ± 0.11	2.18 ± 0.07
Adrenals	0.041 ± 0.004	0.037 ± 0.003	0.042 ± 0.002	0.042 ± 0.002	0.040 ± 0.007	0.040 ± 0.005	0.040 ± 0.003

Haematology and Clinical chemistry - At one day after the last treatment the data indicated effects on the immune system as the total white blood cell (WBC) count was decreased (Table A.6). There was an apparent increase in the percentage of neutrophilic granulocytes in the blood. This increase in the percentage of neutrophils did not actually represent an increase in an absolute number of neutrophilic granulocytes. Instead these remained static as the absolute total WBC number decreased. In addition, in the animals treated with 64 mg/kg CuO some red blood cell parameters (the number of red blood cells, haemoglobin content, and haematocrit) were decreased. At day 21 after treatment (day 26) all white and red blood cell parameters were similar to the values of the control vehicle-treated animals (Table A.7). There was no treatment-related effect in the cellularity of the bone marrow after treatment with CuO nanoparticles (Table A.8).

Table A. 6 Bone marrow cellularity in male rats after 5 days consecutive oral administration of CuO.

CuO								Pilot Study	Extra Study
Dose	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg	512 mg/kg
Day 6									
WBC x10^9/L	8.56 ± 2.67	9.45 ± 4.80	4.79 ± 2.99	12.78 ± 11.86	11.35 ± 8.16	8.19 ±2.45	7.94 ± 5.50	13.72 ± 13.26 (2)	10.45 ± 4.97
Day 26									
WBC x10^9/L	12.91 ± 5.51	13.18 ± 6.91 (3)	12.20 ± 6.53	11.19 ± 7.56 (3)	6.11 ± 3.01	12.10 ± 2.44 (3)	12.36 ± 2.01	NP	NP

Table A. 7 Haematology in male rats after 5 days consecutive oral administration of CuO - At day 6 (24 hours after the last administration).

CuO								Pilot Study	Extra Study
Dose	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg	512 mg/kg
WBC	5.07 ±	4.46 ±	5.27 ±	**3.92 ± 0.40*b**	4.40 ±	4.39 ±	**3.98 ± 0.24***	**2.89 ± 0.41****	9.53 ±
x10⁹/L	0.53	0.52	0.7		0.41	0.51			1.47
RBC	8.03 ±	7.96 ±	8.00 ±	8.12 ±	7.84 ±	7.81 ±	7.68 ±	**7.50 ± 0.12***	8.33 ±
x10¹²/L	0.29	0.15	0.12	0.10	0.27	0.12	0.21		0.92
HgB	8.60 ±	8.38 ±	8.60 ±	8.65 ±	8.43 ±	8.33 ±	8.05 ±	**8.07 ± 0.19***	9.25 ±
Mmol/L	0.36	0.40	0.16	0.13	0.26	0.15	0.21		0.85
Hct	0.415	0.403	0.410	0.420	0.402	0.403	0.400	**0.380 ± 0.00***	0.218
L/L	± 0.02	± 0.02	± 0.01	± 0.02	± 0.02	± 0.01	± 0.00		± 0.02
MCV	52 ±	50 ±	51 ±	51 ±	52 ±	52 ±	52 ±	51 ±	52 ±
fl	0.7	1.7	0.5	0.6	0.9	0.4	1.3	0.1	1.6
MCH	1.07 ±	1.05 ±	1.08 ±	1.07 ±	1.07 ±	1.07 ±	1.05 ±	1.07 ±	1.12 ±
fmol	0.01	0.03	0.02	0.01	0.01	0.01	0.00	0.01	0.02
MCHC	20.62	20.79	20.97	20.68	20.76	20.60	20.46	20.77	21.24
Mmol/L	± 0.22	± 0.20	± 0.25	± 0.24	± 0.31	± 0.17	± 0.48	± 0.17	± 0.33
RDW	11.41	11.69	11.59	11.44	11.36	11.44	11.58	11.60	11.96
%	± 0.19	± 0.59	± 0.19	± 0.18	± 0.19	± 0.18	± 0.11	± 0.14	± 0.21
HDW	1.45 ±	1.50 ±	1.51 ±	1.47 ±	1.43 ±	1.48 ±	1.43 ±	1.43 ±	1.6+7
Mmol/L	0.06	0.04	0.01	0.02	0.04	0.07	0.10	0.05	± 0.08
PLT	739 ±	577 ±	730 ±	728 ±	739 ±	692 ±	766 ±	739 ±	1196 :
x10⁹/L	36	204	32	18	9	146	41	45	236
MPV	6.70 ±	7.56 ±	6.90 ±	6.46 ±	6.90 ±	7.15 ±	6.45 ±	6.82 ±	7.56 ±
fl	0.23	0.97	0.25	0.29	0.43	0.95	0.42	0.26	0.70
Neutrophils	9.9 ±	13.0 ±	11.1 ±	8.8 ±	11.7 ±	13.0 ±	11.2 ±	15.0 ±	27.5 ±
%	0.6	3.0	1.7	1.1	0.8	2.1	1.1	2.1	13.6
Lymphocytes	86.5 ±	80.8	84.7 ±	85.5 ±	83.0 ±	81.4 ±	**83.0 ± 0.8***	81.4 ±	67.2 ±
%	0.7	±4.6	2.4	2.0	1.0	2.8		2.6	15.4
Monocytes	1.54 ±	1.80 ±	2.01 ±	1.66 ±	1.37 ±	1.71 ±	1.78 ±	1.52 ±	2.56 ±
%	0.38	0.54	0.14	0.21	0.13	0.20	0.32	0.26	1.11
Eosinophils	1.61 ±	3.99 ±	1.66	3.65 ±	3.76 ±	3.56 ±	**3.68 ± 0.18****	1.80 ±	0.64 ±
%	0.70	1.51	0.69	1.96	0.32	1.62		0.80	0.38
LUC	0.30 ±	0.33 ±	0.46 ±	0.41 ±	0.23 ±	0.25 ±	0.35 ±	0.18 ±	2.01 ±
%	0.12	0.15	0.08	0.11	0.05	0.07	0.28	0.02	1.38
Basophils	0.05 ±	006 ±	0.05 ±	0.03 ±	0.09 ±	0.06 ±	0.05 ±	0.10 ±	0.08 ±
%	0.04	0.06	0.10	0.05	0.04	0.05	0.00	0.09	0.03
Reticulocytes	2.70 ±	2.69 ±	2.85 ±	2.49 ±	2.47 ±	2.98 ±	2.57 ±	2.07 ±	1.66 ±
%	0.41	0.44	0.11	0.34	0.25	0.38	0.5	0.30	1.04
Neutrophils	0.50 ±	0.58 ±	0.59 ±	0.35 ±	0.51 ±	0.58 ±	0.44 ±	0.44 ±	2.49 ±
x10⁹/L	0.08	0.15	0.16	0.07	0.07	0.16	0.01	0.13	1.09
Lymphocytes	4.39 ±	3.62 ±	4.45 ±	3.36 ±	3.64 ±	3.56 ±	**3.30 ± 0.23***	**2.35 ± 0.26****	6.56 ±
x10⁹/L	0.44	0.53	0.48	0.38	0.33	0.31			2.54
Monocytes	0.08 ±	0.08 ±	0.11 ±	0.07 ±	0.06 ±	0.08 ±	0.07 ±	0.04 ±	0.23 ±
x10⁹/L	0.02	0.02	0.02	0.01	0.01	0.02	0.01	0.00	0.09
Eosinophils	0.08 ±	0.17 ±	0.09 ±	0.14 ±	0.16 ±	0.16 ±	0.15 ±	0.05 ±	0.06 ±
x10⁹/L	0.04	0.05	0.05	0.07	0.00	0.07	0.02	0.03	0.03
LUC	0.01 ±	0.01 ±	0.02 ±	0.02 ±	0.01 ±	0.01 ±	0.02 ±	0.00 ±	0.01 ±
x10⁹/L	0.01	0.01	0.00	0.01	0.00	0.01	0.01	0.00	0.01
Basophils	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.18 ±
x10⁹/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.10
Reticulocytes	0.22 ±	0.21 ±	0.23 ±	0.20 ±	0.19 ±	0.23 ±	0.20 ±	0.16 ±	0.13 ±
x10¹²/L	0.03	0.03	0.01	0.03	0.01	0.03	0.02	0.02	0.07

Table A. 8 Haematology in male rats after 5 days consecutive oral administration of CuO - At day 26 (21 days after the last administration).

CuO Dose	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg
WBC	5.40 ±	5.95 ±	5.15 ±	4.87 ±	6.44 ±	5.60 ±	5.33 ±
$x10^9$/L	0.99	1.18	0.31	0.80	0.96	1.13	0.99
RBC	8.54 ±	8.55 ±	8.59 ±	8.66 ±	8.69 ±	8.77 ±	8.28 ±
$x10^{12}$/L	0.25	0.06	0.16	0.27	0.15	0.31	0.96
HgB	8.73 ±	8.57 ±	8.55 ±	8.73 ±	8.85 ±	8.87 ±	8.44 ±
Mmol/L	0.22	0.06	0.29	0.31	0.25	0.31	0.83
Hct	0.410	0.413	0.405	0.413	0.425	0.427	0.398
L/L	± 0.01	± 0.01	± 0.01	± 0.01	± 0.01	± 0.01	± 0.04
MCV	49 ±	48 ±	47 ±	47 ±	49 ±	48 ±	48 ±
fl	0.5	0.4	0.3	0.6	0.3	0.1	0.8
MCH	1.02 ±	1.01 ±	1.00 ±	1.01 ±	1.02 ±	1.01 ±	1.02 ±
fmol	0.02	0.02	0.01	0.01	0.01	0.01	0.02
MCHC	20.95	20.92	21.00	21.26	20.94	21.07	21.21
Mmol/L	± 0.14	± 0.14	± 0.28	± 0.27	± 0.14	± 0.07	± 0.19
RDW	11.49	11.55	11.59	11.55	11.48	11.57	11.59
%	± 0.15	± 0.09	± 0.14	± 0.22	± 0.12	± 0.15	± 0.19
HDW	1.71 ±	1.74 ±	1.73 ±	1.80 ±	1.69 ±	1.75 ±	1.69 ±
Mmol/L	0.03	0.01	0.03	0.05	0.03	0.03	0.04
PLT	707 ±	714 ±	693 ±	706 ±	678 ±	681 ±	749 ±
$x10^9$/L	20	28	26	23	60	19	69
MPV	7.14 ±	6.98 ±	6.93 ±	7.15 ±	7.11 ±	7.23 ±	6.83 ±
fl	0.29	0.24	0.19	0.44	0.28	0.51	0.17
Neutrophils	11.4 ±	12.0 ±	10.8 ±	10.1 ±	12.0 ±	11.6 ±	10.8 ±
%	1.3	0.2	2.0	1.3	2.1	2.9	2.4
Lymphocytes	84.1 ±	84.0 ±	85.0 ±	86.6 ±	84.0 ±	85.0 ±	83.5 ±
%	1.8	1.1	2.6	2.5	2.4	3.5	4.2
Monocytes	1.41 ±	2.00 ±	1.88 ±	1.60 ±	1.78 ±	1.47 ±	1.85 ±
%	0.06	0.43	0.48	0.40	0.46	0.38	0.48
Eosinophils	2.68 ±	1.43 ±	1.66 ±	1.35 ±	1.75 ±	1.33 ±	3.25 ±
%	2.08	0.68	0.21	0.74	0.26	0.25	2.51
LUC	0.39 ±	0.45 ±	0.58 ±	0.38 ±	0.45 ±	0.55 ±	0.42 ±
%	0.22	0.13	0.21	0.16	0.19	0.26	0.31
Basophils	0.06 ±	0.07 ±	0.03 ±	0.08 ±	0.04 ±	0.07 ±	0.12 ±
%	0.03	0.03	0.03	0.03	0.05	0.06	0.04
Reticulocytes	2.15 ±	2.22 ±	2.12 ±	2.17 ±	2.00 ±	2.05 ±	2.24 ±
%	0.16	0.18	0.06	0.27	0.12	0.20	0.43
Neutrophils	0.62 ±	0.72 ±	0.56 ±	0.48 ±	0.76 ±	0.67 ±	0.59 ±
$x10^9$/L	0.17	0.14	0.12	0.07	0.04	0.30	0.24
Lymphocytes	4.54 ±	5.01 ±	4.38 ±	4.22 ±	5.43 ±	4.73 ±	4.43 ±
$x10^9$/L	0.86	1.06	0.21	0.75	0.97	0.76	0.62
Monocytes	0.08 ±	0.12 ±	0.10 ±	0.08 ±	0.11 ±	0.08 ±	0.09 ±
$x10^9$/L	0.01	0.01	0.03	0.02	0.01	0.03	0.00
Eosinophils	0.14 ±	0.08 ±	0.08 ±	0.07 ±	0.12 ±	0.08 ±	0.19 ±
$x10^9$/L	0.08	0.02	0.02	0.03	0.03	0.03	0.17
LUC	0.02 ±	0.03 ±	0.03 ±	0.02 ±	0.03 ±	0.03 ±	0.02 ±
$x10^9$/L	0.02	0.01	0.02	0.01	0.01	0.02	0.01
Basophils	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.00 ±	0.00 ±
$x10^9$/L	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Reticulocytes	0.18 ±	0.19 ±	0.18 ±	0.19 ±	0.17 ±	0.18 ±	0.18 ±
$x10^{12}$/L	0.01	0.01	0.00	0.02	0.01	0.02	0.01

The results of the clinical chemistry are presented in Table A.9. At day 6 alterations in the level of alkaline phosphatase and aspartate aminotransferase enzymes indicated the presence of liver toxicity. At the highest dose of 64 mg/kg lactate dehydrogenase levels were also increased indicating cell and organ damage. Most of the alterations were no longer present at day 21 after treatment. Animals treated with 512 mg/kg showed similar alterations in clinical chemistry (low level of alkaline phosphatase, high level of aspartate aminotransferase, and high level of lactate dehydrogenase) thus supporting the data of the dose-response study.

Table A. 9 Clinical chemistry in male rats after 5 days consecutive oral administration of CuO - at day 26 (21 days after the last administration.

CuO Dose	0 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg
TP g/L	49 ± 1	49 ± 1	49 ± 2	50 ± 1 (3) <DL(1)	51 ± 1	50 ± 1	52 ± 1
ALB g/L	13.6 ± 0.2	13.5 ± 0.6	13.2 ± 0.3	13.6 ± 0.3 (3) <DL(1)	14.1 ± 0.3	13.8 ± 0.3	13.8 ± 0.3
ALP IU/L	276 ± 19	290 ± 21	**232 ± 19***	209 ± 62	274 ± 30	241 ± 29	236 ± 44
ALT IU/L	49 ± 4.9	52 ± 4.0	46 ± 4.1	43 ± 5.4	50 ± 5.9	51 ± 2.1	46 ± 2.0
AST IU/L	64 ± 3.7	70 ± 6.7	62 ± 1.4	63 ± 4.1	66 ± 3.7	65 ± 4.4	68 ± 6.0
GGT U/L	0.44 ± 0.2 (2) <DL(2)	0.51 ± 0.3 (3) <DL(1)	0.15 ± 0.1	0.27 ± 0.1	0.73 ± 0.3 (2) <DL(2)	0.26 ± 0.1 (3) <DL(1)	0.54 ± 0.1 (2) <DL(2)
LDH IU/L	635 ± 129	634 ± 316	585 ± 223	694 ± 156	547 ± 242	573 ± 205	822 ± 131
CHOL mmol/L	1.26 ± 0.06	1.26 ± 0..02	1.26 ± 0.09	1.26 ± 0.10	1.24 ± 0.05	1.30 ± 0.10	1.40 ± 0.20
TG mmol/L	1.21 ± 0.15	1.17 ± 0.20	1.27 ± 0.30	1.22 ± 0.18 (3) <DL()1)	1.49 ± 0.20	1.08 ± 0.22	1.41 ± 0.26
CRE µmol/L	17.5 ± 1.1	18.5 ± 2.6	19.2 ± 1.2	20.9 ± 1.8 (3) <DL(1)	20.4 ± 3.4	19.9 ± 2.3	22.9 ± 5.4
GLU mmol/L	10.8 ± 1.2	10.2 ± 0.8	10.2 ± 0.3	10.6 ± 0.7	10.4 ± 0.6	10.1 ± 1.1	10.9 ± 0.3
K mmol/L	4.8 ± 0.1	4.8 ± 0.2	5.0 ± 0.1	4.8 ± 0.2	4.9 ± 0.1	4.9 ± 0.3	4.8 ± 0.3
Ca mmol/L	2.58 ± 0.06	2.49 ± 0.05	2.53 ± 0.05	2.70 ± 0.29	2.52 ± 0.02	2.55 ± 0.06	2.54 ± 0.06
Cl mmol/L	98 ± 0.7	100 ± 1.2	99 ± 0.5	98 ± 0.8 (3)	98 ± 1.3	98 ± 0.8	99 ± 1.0
Fe µmol/L	36.3 ± 1.9	33.4 ± 2.1	35.4 ± 1.4	33.8 ± 5.7	35.3 ± 2.0	36.7 ± 1.4	40.1 ± 4.2
Na mmol/L	142 ± 0.7	141 ± 1.0	141 ± 0.6	140 ± 0.3 (3)	141 ± 0.4	141 ± 1.3	142 ± 1.5
Urea mmol/l	6.2 ± 0.2	6.1 ± 0.3	5.8 ± 0.3	6.8 ± 1.7	7.1 ± 0.3	6.1 ± 0.4	6.6 ± 1.6
Uric Acid	43 ± 10.4	39 ± 8.2	43 ± 5.3	49 ± 19.8	41 ± 4.5	47 ± 10.0	45 ± 7.2
TBAX µmol/L	43 ± 5	26 ± 17	33 ± 13	34 ± 17	50 ± 13	45 ± 18	31 ± 12
TBIL µmol/L	7.1 ± 1.0	8.4 ± 1.0	7.8 ± 0.8	9.7 ± 3.6	7.5 ± 0.7	8.3 ± 1.0	7.7 ± 0.9
ROM IU/L	265 ± 28	258 ± 7	255 ± 12	268 ± 34	266 ± 16	260 ± 14	288 ± 35
SHP µmol/L	260 ± 17	272 ± 11	233 ± 11	216 ± 54	276 ± 13	240 ± 9	244 ± 17

Histopathology - There were no test item-related microscopic observations in the initial dose-response study up to a dose of 64 mg/kg (data not shown). All of the recorded microscopic findings were within the range of background pathology encountered in rats of this age and strain. There was no test item-related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations in the initial dose-response study with doses up to 64 mg/kg. For animals treated in the additional study with a dose of 512 mg/kg and evaluated at day, 6 alterations were observed in stomach, liver, and bone marrow.

- Bone marrow: Increased myeloid elements/decreased erythroid elements in the bone marrow of 1/3 rats (slight).

- Stomach: An increased incidence and severity of submucosal glandular inflammation with eosinophilic granulocytes in 4/4 rats treated with 512 mg/kg CuO (1 slight, 3 moderate) compared to 2/4 control rats (minimal).

- Liver: Kupffer cell hypertrophy/hyperplasia in 2/4 rats (slight), inflammation (mixed inflammatory cells) in 2/4 rats (1 moderate, 1 marked), hepatocellular hypertrophy in 3/4 rats (2 minimal, 1 moderate), hepatocellular necrosis in 3/4 rats (1 slight, 1 moderate, 1 marked) and single cell necrosis (above the background level of minimal) in 2/4 rats (1 moderate, 1 marked).

The histopathological observations in animals treated with 512 mg/kg confirm the effects seen in the clinical chemistry parameters. These clinical chemistry effects were already observed at doses for which no histopathological alterations were observed (up to 64 mg/kg CuO).

Cu content of tissues - Cu content of tissues after 5 consecutive days of CuO oral administration is presented in Table A.10. For the liver and mesenteric lymph node (MLN) also at the lower doses investigates (32 and 64 mg/kg) an increase in Cu content was noted. For the highest dose investigated (512 mg/kg) no statistical evaluation could be performed as contemporary controls were lacking. However, besides the liver and MLN also in the kidney, a clear increase was present when compared to vehicle-treated animals in the earlier experiment. For Thymus and spleen, the data indicate a trend for an increase at 512 mg/kg. At day 26 the increased levels of Cu in liver and kidney were returned to levels in the control animals range.

Table A. 10 Cu in organs of male rats after 5 days consecutive oral administration of CuO.

Day 6 Treatment dose	Organ Liver	Lung	Kidney	Spleen	MLN	Thymus	Testis	Brain cortex
0 mg/kg control	13 ± 0.4[a]	6 ± 1	41 ± 6	6 ± 0.4	5 ± 1	4 ± 0.3	11 ± 0.3	9 ± 0.1
32 mg/kg	**42 ± 15.6***	6 ± 1	62 ± 14	6 ± 0.3	8 ± 2	5 ± 1 (3)	11 ± 0.1	9 ± 0.5
64 mg/kg	**75 ± 4.5 (2)***	5 (1)	64 (1)	6 ± 1 (2)	**10 ± 0.3 (2)*****	8 ± 4 (2)	11 ± 0.3 (2)	Nd
512 mg/kg	914 ± 541	70 ± 69	76 ± 42	10 ± 5	26 ± 11	10 ± 7	12 ± 1	10 ± 1
Day 26								
0 mg/kg control	14 ± 0.3	7 ± 0.2	62 ± 9	6 ± 0.6	4 ± 2	5 ± 1	12 ± 1	10 ± 3
32 mg/kg	12 ± 1.5	6 ± 1.3	52 ± 4 (3)	5 ± 0.9	5 ± 2	5 ± 2	11 ± 1	9 ± 0.4

Cytokine protein – Figure A.13 below shows the cytokine concentrations detected in each of the tissues examined following CuO treatment. Analysis of variance of the data indicated that there was no significant increase or decrease in the amount of each cytokine at the doses chosen when compared with the control. There was no IL-13 detected in any of the tissues examined. Only IL-1α and IL-1β were detected in all of the tissues; there was no evidence of an effect of dose on cytokine production. IL-6 was detected only in liver tissue, and both IL-12 and TNF-α were detected in stomach, colon and liver. There was no significant effect of oral CuO treatment at either dose investigated compared with the control treated animals.

Oral Study CuO

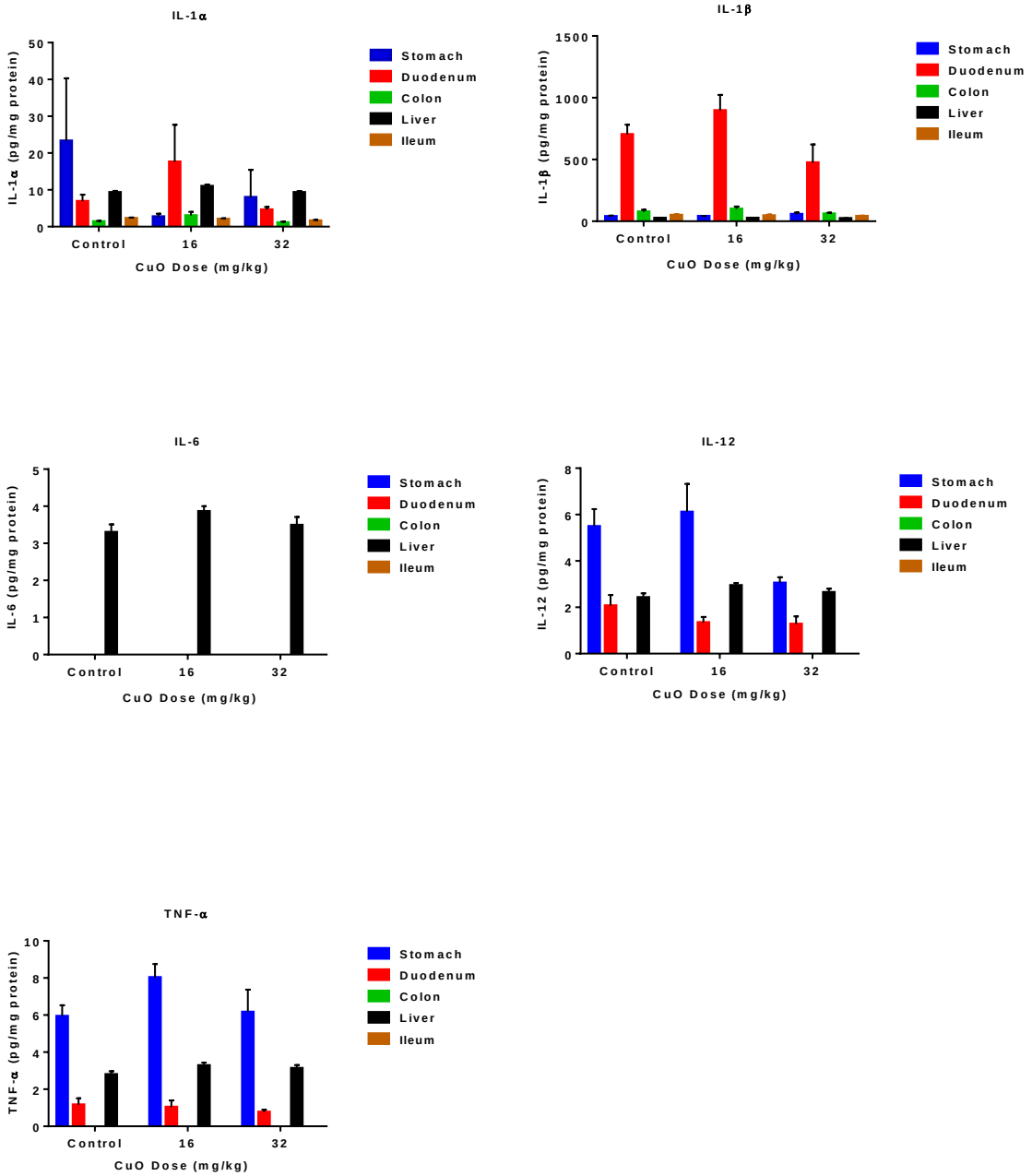


Figure A. 13 cytokine protein concentrations (µg/mg protein) present in selected tissues from rats dosed orally with 16 and 32mg/kg of CuO and sacrificed at day 6 after oral treatment for 5 consecutive days (days 1-5). Data represent the mean±SEM of each cytokine detected (µg/mg protein) and represents data from four separate animals.

GSH and reduced GSH in the liver - After CuO treatment, there was a significant increase in the total GSH in the livers of animals treated with 16 and 32mg/kg ($p<0.01$ and $p<0.001$ respectively) compared with controls. In contrast, in animals treated with 512mg/kg, there was a significant decrease ($p<0.05$) in the amount of total GSH. In animals treated with 32 and 512mg/kg CuO, there was a significant increase in the amount of reduced GSH ($p<0.05$ and $p<0.01$ respectively) compared with controls (Figure A.14).

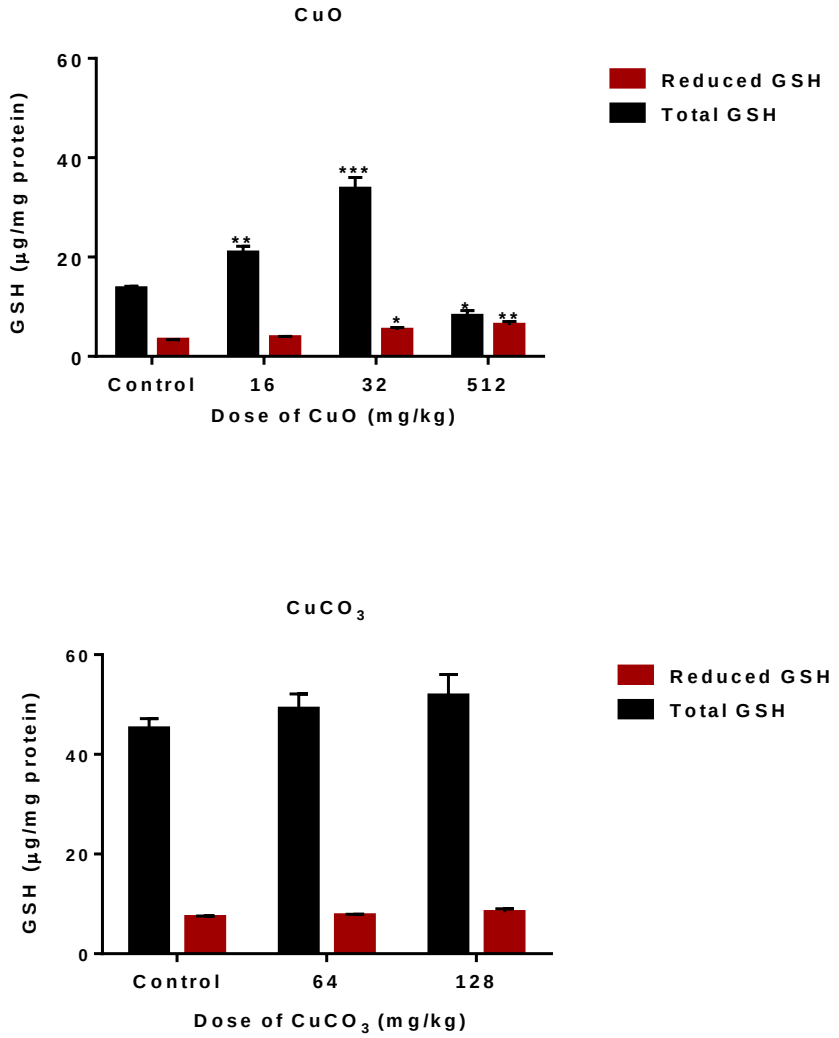


*Figure A. 14 GSH concentration (total and reduced forms) (µg/mg protein) present in the liver of rats dosed orally with 16, 32 and 512mg/kg of CuO and 64 and 128mg/kg of $CuCO_3$. Animals were sacrificed at day 6 after oral treatment for 5 consecutive days (days 1-5). Data represent the mean±SEM of GSH (µg/mg protein) and represents data from four separate animals. (*p<0.05; **p<0.01; ***p<0.001 compared with the control).*

Conclusions on oral CuO administration

For some of the parameters the dose-response study with lower doses (up tp 32 and 64 mg/kg) some indications for liver toxicity was noted. The investigational high dose (512 mg/kg) study group that was later added confirmed the occurring liver toxicity, also confirmed by histopathology. Table 8 shows a further statistical evaluation of the outcomes of the oral CuO studies by using the benchmark dose (DMB) approach. This analysis shows that only for effects on the liver enzyme AST and the red blood cell parameters an effect could be demonstrated. The histopathology confirms the indication for liver toxicity by the effect on the AST enzyme, as it showed liver lesions after oral exposure to CuO at high doses.

Results: $CuCO_3$ - General toxicity

For $CuCO_3$ a dose range finding study was performed according to OECD 425 (Acute oral toxicity, Up and Down Procedure). The dose according to OECD 425 was administered once, and the animals were followed by signs of toxicity. Toxicity signs for evaluation were: pain, abnormal behaviour, isolation, diarrhoea, exudation, breathing, and severe weight loss and death. The dose of 128 mg/kg was found to be suitable for oral administration.

Repeated (5 times) oral administration of a dose of 128 mg/kg $CuCO_3$ induced severe toxic responses in the treated animals as indicated by the behaviour of the animals, frequent washing and piloerection. Based on these observations the animals scheduled for prolonged observation (autopsy after a recovery period at day 21 after treatment) were autopsied prematurely at days 6 and 7, respectively 24 and 48 hours after the last (day 5) treatment. Table A.11 shows the body and organ weights at day 6 and 7. At day 6 a decrease in body weight was present for the group of animals that were treated with a dose of 128 mg/kg body weight. This was mainly observed for the "recovery" group, although there was no difference between the treatment or recovery durations imposed on animals in each group at this time point. Heart, liver, spleen and thymus showed a decrease in weight, whereas adrenal weights were increased, the latter probably indicating a stress response due to the toxicity of the $CuCO_3$.

Table A. 11 Body and organ weight in male rats after 5 days consecutive oral administration of $CuCO_3$ - At day 6 (24 hours after the last administration).

CuCO3 Dose	0 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg	128 mg/kg	128 mg/kg[a]
Body weight (g)	345 ± 11	385 ±24	394 ± 58	367 ± 15	362 ±11	344 ± 29	327 ± 73	**290 ± 16****
Lung	1.48 ± 0.11	1.56 ± 0.15	1.50 ± 0.04	1.50 ± 0.08	1.36 ± 0.35	1.51 ± 0.53	1.46 ± 0.17	1.34 ± 0.16
Heart	1.210 ± 0.05	1.279 ± 0.07	1.300 ± 0.08	1.244 ±0.09	1.140 ± 0.06	1.185 ± 0.16	**1.002 ± 0.14***	**0.928 ± 0.02****
Liver	13.40 ± 0.57	15.68 ± 1.52	15.14 ± 1.24	14.79 ± 0.25	14.42 ± 0.90	14.15 ± 2.22	**10.79 ± 1.22****	**10.43 ± 1.33****
Kidney (L+R)	2.23 ± 0.15	2.35 ± 0.14	2.38 ± 0.05	2.26 ± 0.11	2.21 ± 0.25	2.29 ± 0.30	2.00 ± 0.75	2.14 ± 0.45
Spleen	0.992 ± 0.09	1.040 ± 0.16	1.032 ± 0.20	0.955 ± 0.09	1.020 ± 0.26	1.059 ± 0.15	**0.508 ± 0.08****	**0.540 ± 0.16****
Thymus	0.706 ± 0.13	0.789 ± 0.10	0.826 ± 0.04	0.745 ± 0.16	0.798 ± 0.21	0.717 ± 0.19	**0.221 ± 0.06****	**0.192 ± 0.06****
Mesenteric Lymph Node	0.254 ± 0.06	0.265 ± 0.05	0.227 ± 0.03	0.188 ± 0.03	0.263 ± 0.03	0.224 ± 0.08	0.210 ± 0.04	0.222 ± 0.06
Testes (L+R)	3.33 ± 0.23	3.42 ± 0.37	3.26 ± 0.38	2.66 ± 0.17	3.11 ± 0.28	3.29 ± 0.37	3.02 ± 0.29	3.06 ± 0.34
Brain	1.92 ± 0.03	1.94 ± 0.05	1.88 ± 0.02	1.93 ± 0.08	1.88 ± 0.11	1.91 ± 0.08	1.86 ± 0.07	1.84 ± 0.11
Adrenals	0.043 ± 0.004	0.051 ± 0.009	0.051 ± 0.006	0.051 ± 0.006	0.052 ± 0.005	0.051 ± 0.004	**0.071 ± 0.015***	**0.063 ± 0.008****

Haematology and Clinical chemistry - An overview of haematological parameters after five-day oral administration of $CuCO_3$ nanoparticles is presented in Table A.12. At day 6 an increase in WBC was present for doses of 32 mg/kg. The increase in WBC was due to an absolute (and also relative) increase in neutrophilic granulocytes. The decrease in a relative number of lymphocytes can be attributed to the increase in neutrophilic granulocytes. The absolute number of lymphocytes was not affected by the $CuCO_3$ nanoparticles treatment. Data for day 26 are shown in Table A.13. At day 26 no alterations in haematological parameters were observed.

Table A. 12 Hematology in male rats after 5 days consecutive oral administration of CuCO3 - At day 6 (24 hours after the last administration).

CuCO3								
Dose	0 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg	128 mg/kg	128 mg/kg[a]
WBC	8.29 ±	13.10 ±	11.46 ±	10.78 ±	13.48 ±	**16.37**	**17.49**	18.35 ±
x10⁹/L	1.56 (3)	1.75	0.55	1.72	3.13	**± 4.85***	**± 7.8***	6.8
RBC	7.32 ±	7.57 ±	7.27 ±	7.20 ±	7.32 ±	7.51 ±	**9.36 ±**	8.38 ±
x10¹²/L	0.26	0.3	0.4	0.35	0.36	0.25	**1.5****	0.17
HgB	8.23 ±	8.48 ±	8.45 ±	8.30 ±	8.20 ±	8.68 ±	**10.0 ±**	9.43 ±
Mmol/L	0.4	0.56	0.31	0.35	0.39	0.22	**0.92****	0.21
Hct	0.40 ±	0.41 ±	0.41 ±	0.40 ±	0.40 ±	0.42 ±	**0.48 ±**	0.45 ±
L/L	0.02	0.03	0.01	0.02	0.02	0.01	**0.05****	0.01
MCV fl	54 ± 1	54 ± 1	57 ± 3	55 ± 1	54 ± 1	55 ± 1	52 ± 4	54 ± 2
MCH	1.12 ±	1.12 ±	1.17 ±	1.15 ±	1.12 ±	1.16 ±	1.08 ±	1.12 ±
fmol	0.02	0.04	0.06	0.03	0.01	0.04	0.11	0.05
MCHC	20.6 ±	20.7 ±	20.3 ±	20.8 ±	20.6 ±	20.9 ±	20.8 ±	20.8 ±
Mmol/L	0.11	0.23	0.36	0.10	0.11	0.46	0.86	0.29
RDW	12.23 ±	12.75 ±	12.44 ±	12.30 ±	12.44 ±	12.10 ±	13.08 ±	13.37 ±
%	0.37	0.44	0.63	0.55	0.43	0.88	1.44	0.8
HDW	1.62 ±	1.64 ±	1.65 ±	1.68 ±	1.66 ±	1.67 ±	2.01 ±	1.91 ±
Mmol/L	0.04	0.06	0.04	0.06	0.04	0.12	0.28	0.10
PLT	969 ±	956 ±	1160 ±	915 ±	1033	971 ±	1171 ±	1135 ±
x10⁹/L	79	114	172	200	±94	241	102	35
MPV	6.27 ±	6.58 ±	6.28 ±	6.46 ±	6.56 ±	7.16 ±	6.64 ±	6.33 ±
fl	0.50	0.68	0.58	0.25	0.73	1.73	1.35	0.50
Neutrophils	16.4 ±	15.8 ±	13.2 ±	16.0 ±	17.0 ±	16.8 ±	**55.5 ±**	49.5 ±
%	2.6	1.5	4.4	3.2	2.4	3.7	**14.1****	7.4
Lymphocytes	78.9 ±	79.1 ±	82.3 ±	79.5 ±	78.0 ±	77.3 ±	**35.1 ±**	39.4 ±
%	3.2	1.8	5.1	3.3	2.8	4.2	**16****	9.4
Monocytes	2.2 ±	2.7 ±	2.5 ±	2.2 ±	2.7 ±	3.8 ±	**6.0 ±**	7.2 ±
%	0.5	0.5	0.4	0.3	0.5	0.3	**0.7****	0.5
Eosinophils	2.0 ±	1.8 ±	1.5 ±	1.7 ±	1.6 ±	1.4 ±	0.7 ±	0.8 ±
%	0.8	0.4	0.6	0.1	0.6	0.4	0.1	0.4
LUC	0.5 ±	0.5 ±	0.4 ±	0.5 ±	0.6 ±	0.6 ±	2.6 ±	3.2 ±
%	0.3	0.1	0.1	0.1	0.4	0.1	2.1	1.8
Basophils	0.03 ±	0.13 ±	0.09 ±	0.11 ±	0.08 ±	0.10 ±	0.08 ±	0.05 ±
%	0.06	0.05	0.03	0.03	0.05	0.09	0.05	0.00
Reticulocytes	3.29 ±	3.27 ±	4.05 ±	3.65 ±	3.30 ±	1.74 ±	0.85 ±	1.07 ±
%	0.16	0.22	0.25	0.62	0.61	0.25	0.11	0.12
Neutrophils	1.36 ±	2.06 ±	1.53 ±	1.70 ±	2.25 ±	2.69 ±	**10.1 ±**	9.3 ±
x10⁹/L	0.4	0.3	0.6	0.2	0.4	0.7	**6.1****	4.4
Lymphocytes	6.52 ±	10.37 ±	9.42 ±	8.60 ±	10.54 ±	**12.75**	5.59 ±	6.95 ±
x10⁹/L	1.1	1.5	0.4	1.7	2.6	**± 4.1***	1.9	2.3
Monocytes	0.19 ±	0.36 ±	0.28 ±	0.24 ±	0.38 ±	0.61 ±	**1.08 ±**	1.33 ±
x10⁹/L	0.08	0.09	0.05	0.06	0.14	0.15	**0.56****	0.55
Eosinophils	0.17 ±	0.23 ±	0.18 ±	0.18 ±	0.22 ±	0.21 ±	0.12 ±	0.15 ±
x10⁹/L	0.05	0.03	0.08	0.03	0.09	0.06	0.04	0.10
LUC	0.05 ±	0.07 ±	0.06 ±	0.05 ±	0.09 ±	0.11 ±	**0.57 ±**	0.60 ±
x10⁹/L	0.04	0.02	0.01	0.02	0.07	0.05	**0.56***	0.51
Basophils	0.00 ±	0.01 ±	0.01 ±	0.01 ±	0.01 ±	0.02 ±	0.01 ±	0.01 ±
x10⁹/L	0.00	0.01	0.01	0.01	0.01	0.02	0.01	0.01
Reticulocytes	0.24 ±	0.25 ±	**0.29 ±**	0.26 ±	0.24 ±	**0.13 ±**	**0.08 ±**	0.09 ±
x10¹²/L	0.02	0.02	**0.02***	0.04	0.04	**0.02****	**0.01*****	0.01

Table A. 13 Hematology in male rats after 5 days consecutive oral administration of CuCO3 - At day 26 (21 days after the last administration).

CuCO3

Dose	0 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg
WBC	10.50	13.33	10.76	12.31	11.93	9.67 ±
$x10^9$/L	± 1.75	± 2.62	± 1.90	± 1.47	± 2.53	1.38
RBC	7.90 ±	7.94 ±	7.95 ±	7.85	7.83 ±	7.84 ±
$x10^{12}$/L	1.14	0.21	0.34	±0.26	0.21	0.15
HgB	8.40 ±	8.53 ±	8.55 ±	8.38 ±	8.28 ±	8.13 ±
Mmol/L	0.33	0.36	0.41	0.26	0.15	0.33
Hct	0.42 ±	0.41 ±	0.41 ±	0.41 ±	0.41 ±	0.39 ±
L/L	0.02	0.02	0.02	0.01	0.01	0.01
MCV fl	52 ±2	52 ±1	52 ±1	52 ±0	52 ±1	50 ± 2
MCH	1.07 ±	1.07 ±	1.08 ±	1.06 ±	1.06 ±	1.04 ±
fmol	0.05	0.03	0.03	0.01	0.02	0.04
MCHC	20.4 ±	20.7 ±	20.8 ±	20.6 ±	20.4 ±	20.7 ±
Mmol/L	0.1	0.3	0.3	0.2	0.2	0.2
RDW	12.71	13.24	12.64	13.23	13.76	13.61
%	± 0.40	± 0.41	± 0.39	±0.81	± 0.64	± 0.68
HDW	1.79 ±	1.95 ±	1.91	1.90	1.98 ±	1.94
Mmol/L	0.09	0.13	±0.04	±0.09	0.11	±0.06
PLT	915 ±	717 ±	932 ±	981 ±	919 ±	939 ±
$x10^9$/L	69	128	143	48	74	78
MPV	6.58 ±	6.93 ±	7.15 ±	6.71 ±	6.20 ±	6.67 ±
fl	0.53	0.73	1.01	0.24	0.35	0.25
Neutrophils	17.5 ±	19.7 ±	13.4 ±	18.7 ±	20.2 ±	17.7 ±
%	5.2	4.5	2.2	1.3	7.0	3.7
Lymphocytes	79.0 ±	75.4 ±	82.6 ±	77.6 ±	75.2 ±	78.5 ±
%	5.1	3.3	3.5	1.7	6.3	3.4
Monocytes	2.03 ±	3.06 ±	2.40 ±	2.11 ±	2.69 ±	1.76 ±
%	0.41	1.29	1.31	0.44	0.86	0.29
Eosinophils	1.15 ±	1.40 ±	1.26 ±	1.38 ±	1.40 ±	1.60 ±
%	0.25	0.18	0.27	0.29	0.44	0.56
LUC	0.2 ±	0.3 ±	0.3 ±	0.2 ±	0.4 ±	0.3 ±
%	0.1	0.2	0.2	0.0	0.2	0.2
Basophils	0.11 ±	0.10 ±	0.06 ±	0.08 ±	0.10 ±	0.09 ±
%	0.03	0.04	0.05	0.03	0.04	0.02
Reticulocytes	3.07 ±	3.05 ±	2.68 ±	3.38 ±	3.28 ±	3.31 ±
%	0.28	0.42	0.35	0.37	0.40	0.39
Neutrophils	1.86 ±	2.56 ±	1.47 ±	2.29 ±	2.29 ±	1.73 ±
$x10^9$/L	0.75	0.45	0.48	0.24	0.42	0.51
Lymphocytes	8.27 ±	10.10	8.84 ±	9.56 ±	9.08 ±	7.56 ±
$x10^9$/L	1.34	± 2.29	1.20	1.18	2.62	0.91
Monocytes	0.22 ±	0.43 ±	0.27 ±	0.26 ±	0.34 ±	0.17 ±
$x10^9$/L	0.06	0.22	0.18	0.09	0.17	0.05
Eosinophils	0.12 ±	0.19 ±	0.14 ±	0.17 ±	0.16 ±	0.15 ±
$x10^9$/L	0.01	0.05	0.06	0.04	0.04	0.05
LUC	0.03 ±	0.05 ±	0.04 ±	0.02 ±	0.06 ±	0.03 ±
$x10^9$/L	0.01	0.03	0.02	0.00	0.04	0.03
Basophils	0.01 ±	0.01 ±	0.01 ±	0.01 ±	0.01 ±	0.00 ±
$x10^9$/L	0.01	0.01	0.01	0.01	0.01	0.00
Reticulocytes	0.24 ±	0.24 ±	0.21 ±	0.27 ±	0.26 ±	0.26 ±
$x10^{12}$/L	0.02	0.03	0.03	0.02	0.03	0.03

Bone marrow - There was no treatment-related effect in the cellularity of the bone marrow after treatment with $CuCO_3$ nanoparticles (Table A.14). However, by microscopical evaluation of the bone marrow, there was a shift into myeloid cells of the bone marrow that was confirmed by histopathology.

Table A. 14 Bone marrow cellularity in male rats after 5 days consecutive oral administration of $CuCO_3$.

$CuCO_3$ Dose	0 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg	128 mg/kg	128 mg/kg[a]
Day 6								
WBC $x10^9/L$	21.25 ± 6.72	22.90 ± 8.84	22.88 ± 2.27	18.80 ± 11.21	15.90 ± 3.80	14.68 ± 3.37	13.60 ± 2.72	18.09 ±2.09
Day 26								
WBC $x10^9/L$	16.71 ± 4.09	23.29 ± 6.18	18.86 ± 4.63	17.94 ± 8.94	18.82 ± 6.32	17.47 ± 4.09	NP	NP
Erythroid BM cells (%)	45.8 ± 4.3						**16 ± 4.4 (3)***	**22.8 ± 8.7***
Myeloid/ Lymphoid BM cells (%)	54.2 ± 4.3						**84 ± 4.4 (3)***	**77.2 ± 8.7***

Clinical chemistry - The results of the clinical chemistry are presented in Table A.15 and A.16. At day 6 alkaline phosphatase was decreased while aspartate aminotransferase, alanine aminotransferase, and lactate dehydrogenase were increased. These enzyme alterations in the blood indicate both liver and organ toxicity. In addition, free fatty acids, creatinine, iron and potassium levels in blood were increased. At day 26 only some incidental alterations were observed in clinical chemistry parameters, and most parameters were back to levels similar to control vehicle-treated animals.

Table A. 15 Clinical chemistry in male rats after 5 days consecutive oral administration of $CuCO_3$ - At day 6 (24 hours after the last administration).

CuCO3 Dose	0 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg	128 mg/kg	128 mg/kg[a]
TP g/L	55.4 ± 0.6	55.9 ± 0.8	56.5 ± 1.2	54.7 ± 2.2	53.5 ± 1.4	**50.3 ± 2.5***	55.8 ± 3.7 (3)	52.1 ± 4.3 (3)
ALB g/L	15.7 ± 0.6	16.0 ± 0.5	15.9 ± 0.7	16.1 ± 0.8	15.0 ± 0.7	14.2 ± 1.5	15.9 ± 1.8 (3)	14.5 ± 0.9 (3)
ALP IU/L	222 ± 35	256 ± 20	208 ± 16	266 ± 48	238 ± 19	247 ± 51	**80 ± 21****	**116 ± 18****
ALT IU/L	54 ± 2	59 ± 4	57 ± 13	58 ± 10	60 ± 14	**310 ± 94****	409 ± 360	**612 ± 186****
AST IU/L	93 ± 9	96 ± 8	97 ± 15	101 ± 10	113 ± 19	**438 ± 110****	**1224 ± 545***	**1771 ± 1122***
GGT U/L	0.36 ± 0.2 (3) <DL(1)	0.50 ± 0.1 (2) <DL(2)	0.35 ± 0.1 (2) <DL(1)	047 ± 0.3 (2) <DL(2)	0.55 (1) <DL(2)	0.32 ± 0.2 (3) <DL(1)	0.27 ± 0.2 (3) <DL(1)	7.1 (1)
LDH IU/L	2229 ± 468	1796 ± 455	2556 ± 656	2229 ± 710	2754 ± 462	3139 ± 1726	**9406 ± 4903***	3667 ± 1173
FFA Mmol/L	0.17 ± 0.04	0.20 ± 0.1	0.19 ± 0.03	0.19 ± 0.04	0.25 ± 0.04	**0.42 ± 0.2****	**0.65 ± 0.1****	**0.62 ± 0.2****
CHOL mmol/L	1.21 ± 0.1	1.40 ± 0.2	1.32 ± 0.1	1.42 ± 0.2	1.23 ± 0.2	**1.88 ± 0.4***	1.58 ± 0.4	**2.44 ± 0.5****
TG mmol/L	1.70 ± 0.44	1.71 ± 0.37	2.45 ± 0.47	1.97 ± 0.24	1.84 ± 0.29	1.59 ± 0.6	1.23 ± 0.5	1.06 ± 0.28
CRE µmol/L	22.6 ± 2.2	21.7 ± 3.5	23.7 ± 0.9	24.2 ± 2.4	25.0 ± 3.1	23.0 ± 3.2	**190 ± 110 (3)***[3]	56.2 ± 49
GLU mmol/L	11.8 ± 0.8	11.0 ± 0.5	11.6 ± 1.2	11.6 ± 1.1	10.8 ± 0.3	10.2 ± 1.3	**7.1 ± 2.0****	9.6 = 4.0
K mmol/L	4.81 ± 0.2	4.94 ± 0.4	4.90 ± 0.1	4.89 ± 0.4	5.00 ± 0.2	5.24 ± 0.5	**6.10 ± 0.6 (3)***	**5.59 ± 0.2 (3)***
Ca mmol/L	2.49 ± 0.04	**2.60 ± 0.04***	**2.57 ± 0.03***	2.59 ± 0.06	**2.55 ± 0.01***	2.53 ± 0.02	2.56 ± 0.18	2.53 ± 0.15
Cl mmol/L	101 ± 1.5	100 ± 0.4	101 ± 0.9	100 ± 2	101 ± 0.7	**104 ± 0.8***	99 ± 0.9 (3)	102 ± 1.5 (3)
Fe µmol/L	30.9 ± 2	36.3 ± 4	**39.7 ± 6***	**51.3 ± 11***	**64.8 ± 19***	43.3 ± 24	**66.5 ± 23***	51.6 ± 33
Na mmol/L	142 ± 0.5	141 ± 0.5	142 ± 2.0	143 ± 3.3	142 ± 0.6	143 ± 1.8	137 ± 3.7 (3)	140 ± 2.4 (3)
Urea mmol/l	6.06 ± 1.0	6.44 ± 0.7	5.78 ± 1.2	6.48 ± 0.6	6.24 ± 0.7	5.47 ± 0.6	25.8 ± 14.5	13.3 ± 5.3
Uric Acid	69 ± 27	53 ± 4	74 ± 12	50 ± 5	66 ± 7	55 ± 6	**151 ± 49***	119 ± 58
TBIL µmol/L	5.96 ± 0.19	5.33 ± 0.62	5.55 ± 0.66	5.75 ± 0.5	6.38 ± 0.75	6.11 ± 0.4	13.73 ± 9.66	12.92 ± 7.65
ROM IU/L	292 ± 30	280 ± 22	256 ± 14	261 ± 8	246 ±16	332 ± 35	406 ± 189	360 ± 47
SHP µmol/L	323 ± 41	373 ± 27	335 ± 16	354 ± 20	328 ± 13	277 ± 36	197 ± 160	**148 ± 112***

Table A. 16 Clinical chemistry in male rats after 5 days consecutive oral administration of $CuCO_3$ - at day 26 (21 days after the last administration).

CuCO3 Dose	0 mg/kg	4 mg/kg	8 mg/kg	16 mg/kg	32 mg/kg	64 mg/kg
TP g/L	58.6 ± 2.4	56.9 ± 2.7	56.4 ± 3.0	59.4 ± 1.7	57.5 ± 1.9	57.1 ± 1.6
ALB g/L	16.3 ± 0.7	15.5 ± 1.0	15.3 ± 1.1	16.8 ± 0.2	16.6 ± 0.5	16.0 ± 1.3
ALP IU/L	205 ± 18	188 ± 24	178 ± 31	229 ± 40	197 ± 28	256 ± 39
ALT IU/L	63.7 ± 4.7	70.4 ± 9.6	**48.5 ± 3.8****	69.3 ± 13.1	66.0 ± 8.3	87.2 ± 32.3
AST IU/L	120 ± 7	110 ± 9	**92 ± 6****	121 ± 10	112 ± 5	153 ± 55
GGT U/L	0.71 (1) <DL(3)	0.49 ± 0.0 (2) <DL(1)	1.11 (1) <DL(3)	0.44 (1) <DL(3)	0.67 ± 0.3 (2) <DL(2)	0.69 ± 0.2 (3) <DL(1)
LDH IU/L	3271 ± 548	**2108 ± 333***	**1878 ± 455***	2254 ± 688	2217 ± 657	2488 ± 636
CHOL mmol/L	1.32 ± 0.2	1.39 ± 0.1	1.47 ± 0.1	1.40 ± 0.1	1.14 ± 0.1	1.28 ± 0.1
TG mmol/L	2.20 ± 0.89	2.58 ± 0.80	2.27 ± 0.65	2.84 ± 1.03	2.19 ± 0.91	1.80 ± 0.28
CRE µmol/L	27.2 ± 2.1	28.4 ± 1.8	28.0 ± 2.3	31.6 ± 2.7	29.4 ± 2.3	**31.2 ± 1.5***
GLU mmol/L	11.3 ± 1.8	10.8 ± 1.3	13.8 ± 2.5	11.4 ± 0.8	11.1 ± 0.7	12.3 ± 1.1
K mmol/L	4.84 ± 0.3	5.08 ± 0.3	4.80 ± 0.3	4.94 ± 0.4	4.86 ± 0.3	4.74 ± 0.1
Ca mmol/L	2.50 ± 0.03	2.55 ± 0.05	2.49 ± 0.07	2.50 ± 0.04	2.53 ± 0.05	2.47 ± 0.06
Cl mmol/L	102 ± 1.3	102 ± 1.1	102 ± 1.4	101 ± 1.5	102 ± 1.7	102 ± 1.0
Fe µmol/L	30.5 ± 3.9	31.1 ± 7.7	29.9 ± 3.0	29.5 ± 4.2	30.8 ± 5.9	29.0 ± 3.8
Na mmol/L	144 ± 1.1	143 ± 1.9	142 ± 1.5	142 ± 1.4	144 ± 1.0	143 ± 1.6
Urea mmol/l	7.13 ± 0.7	7.28 ± 0.5	6.49 ± 0.9	7.56 ± 0.6	6.59 ± 0.8	7.04 ± 0.8
Uric Acid	48 ± 3	45 ±5 (3) <DL(1)	51 ± 6	62 ± 12	49 ± 5	54 ± 12
TBIL µmol/L	5.38 ± 0.28	5.30 ± 0.69	5.73 ± 0.83	**6.12 ± 0.17****	5.22 ± 0.77	5.01 ± 0.71
Zn µg/dL	119 ± 3	136 ± 6	124 ± 9	125 ± 3	132 ± 7	114 ± 10
ROM IU/L	297 ± 16	272 ± 23	274 ± 26	304 ± 22	279 ± 11	289 ± 23
SHP µmol/L	349 ± 6	324 ± 21	331 ± 25	327 ± 22	328 ± 17	340 ± 39

Histopathology - Test item-related microscopic findings were observed in a number of organs examined after treatment with 64 or 128 mg/kg CuO_3. These findings comprised:

<u>Starting at 64 mg/kg $CuCO_3$:</u>

Stomach: An increased incidence and severity of submucosal glandular inflammation with eosinophilic granulocytes (a minimal degree is considered to be background) on day 6 was observed in –

- 4/4 rats treated at 64 mg/kg (moderate),
- 4/4 rats treated at 128 mg/kg (1 minimal, 3 slight),
- 2/4 control rats (minimal).

After a 3-week treatment-free period the submucosal glandular inflammation with eosinophilic granulocytes was observed to remain in –

- 4/4 rats 2 slight, 2 moderate) treated at 64 mg/kg,
- 4/4 rats (1 minimal, 3 slight) treated at 128 mg/kg,
- 1/4 control rats (minimal).

Liver: Inflammation (mixed inflammatory cells) occurred at day 6 in –

- 3/4 rats treated at 64 mg/kg (2 slight, 1 moderate),
- 1/4 rats at 128 mg/kg (moderate).

Hepatocellular hypertrophy occurred at day 6 in –

- 2/4 rats at 64 mg/kg (1 slight, 1 moderate),
- 2/4 rats at 128 mg/kg (slight).

Hepatocellular necrosis occurred at day 6 in –

- 1/4 rats at 64 mg/kg (minimal),
- 1/4 rats at 128 mg/kg (slight).

Single cell necrosis occurred at day 6 in –

- all rats treated at 64 (1 minimal, 2 slight, 1 moderate),

- all rats treated at 128 mg/kg (1 minimal, 1 slight, 2 moderate).

Increased mitosis in the liver occurred at day 6 in –

- 1/4 rats treated with 64 (moderate),
- 1/4 rats treated at 128 mg/kg (moderate).

In the animals autopsied at day 7 similar lesions were observed consisting of –

- 2/4 rats with Kupffer cell hypertrophy/hyperplasia (moderate),
- 1/4 rats with vacuolation (slight),
- 3/4 rats with inflammation (1 slight, 2 moderate),
- 3/4 rats with single-cell necrosis (1 slight, 1 moderate, 1 marked).

The minimal single cell necrosis in a single rat treated at 64 mg/kg is considered to be background.

At 128 mg/kg $CuCO_3$:

Liver: Kupffer cell hypertrophy/hyperplasia occurred at day 6 in –

- 2/4 rats (1 slight, 1 moderate)

Hepatocellular vacuolation occurred at day 6 in –

- 3/4 rats (slight) treated at 128 mg/kg CuO3. I

In the second group of animals autopsied at day 7 similar findings were observed in rats treated at 128 mg/kg CuO_3 consisting of –

- 2/4 rats with Kupffer cell hypertrophy/hyperplasia (moderate),
- 1/4 rats with vacuolation (slight).

Intestines: Ulcerations/erosions in the colon occurred at day 6 in –

- 2/4 rats (slight) with oedema in 1/4 rats (minimal)

Inflammation occurred at day 6 in the rectum of –

- 1/4 rats (minimal) with edema (slight).

In the second group of animals autopsied at day 7 intestinal findings consisted of ulcerations/erosions and/or edema in –

- the cecum of 1/4 rats (minimal),
- the colon of 1/4 rats (slight),
- the rectum of 3/4 rats (1 minimal, 1 slight, 1 moderate).

In addition, increased apoptosis was observed in

- the duodenum of 3/4 rats (2 slight, 1 moderate),
- the ileum of 1/4 rats (moderate).

Spleen: Lymphoid atrophy was observed in

- the spleen of 2/4 rats (1 minimal, 1 slight) at day 6
- the spleen of 2/4 rats (minimal) at day 7.

Thymus: Lymphoid atrophy was observed in the thymus of 2/4 rats (moderate) at day 7.

Kidneys: Tubular necrosis occurred in 2/4 rats (marked) with hyaline casts in the same 2/4 rats (moderate) at day 6. Also at day 7 tubular degeneration/regeneration was observed in 2/4 rats (moderate) with tubular intranuclear inclusions (moderate).

Testes: Moderate degeneration of germ cells (including slight multinucleated giant cells) and marked spermatid retention was observed in 1/4 rats at day 7.

Seminal vesicles: Decreased fluid was observed in the seminal vesicles of 4/4 rats (slight) at day 6 which was also present at day 7 in 4/4 rats (1 minimal, 2 slight, 1 moderate).

Bone marrow: Increased myeloid elements/increased erythroid elements in the bone marrow of 3/4 rats (2 minimal, 1 moderate) and myeloid hyperplasia was observed in 3/4 rats (slight) at day 7.

Cu content of tissues - Cu content of tissues after 5 consecutive days of $CuCO_3$ oral administration is presented in Table 17. For the liver, kidney and thymus, at the lower dose investigates (64 mg/kg) an increase in Cu content was noted. For the highest dose (128 mg/kg) increases in Cu content were observed in liver, lung, kidney, spleen, thymus, and mesenteric

lymph node. At day 26 the increased levels of Cu in liver and kidney were returned to levels in the control animal range.

Table A. 17 Cu (µg/g tissue) in organs of male rats after 5 days consecutive oral administration of $CuCO_3$.

$CuCO_3$	Day 6			Day 26	
Dose	0 mg/kg	64 mg/kg	128 mg/kg	0 mg/kg	64 mg/kg
Liver	13±0.5[a]	451±58***	1399±307 (8)***	13±1	29±9*
Lung	8±0.4	9±3	259±335 (8)*	7±0.3	7±0.6
Kidney	29±6	55±13 (3)*	810±369 (8)***	36±17	28±4
Spleen	5±0.2	6±0.6	35±26 (7)*	5±0.2	5±0.3
Thymus	4±0.5	6±0.6***	29±20 (8)***	3±0.3	3±0.4
Mesenteric Lymph Node MLN	6±1	7±1	48±23 (8)**	6±3	6±2
Testes	11±0.3	12±0.2*	15±3 (8)**	11±0.2	13±1**
Brain cortex	9±0.2	10±0.4*	11±1 (8)***	9±0.5	10±7

Cytokine protein – Figure A.15 below shows the cytokines which were detected in each of the tissues examined following $CuCO_3$ treatment. In these exposures, doses of 8, 16 and 32mg/kg were not examined in stomach, colon and duodenum tissues. At the highest doses of 64 and 128mg/kg $CuCO_3$, there was a significant ($p<0.001$) increase in IL-1α in the duodenum, liver and colon samples. There was no significant increase in IL-1β in any of the tissues at the highest dose of $CuCO_3$ and no evidence of IL-6 or IL-13 in any tissue samples (data not shown). There was a significant decrease in IL-12 production in liver tissue at the two highest doses of 64 and 128mg/kg ($p<0.001$; $p<0.01$ respectively).

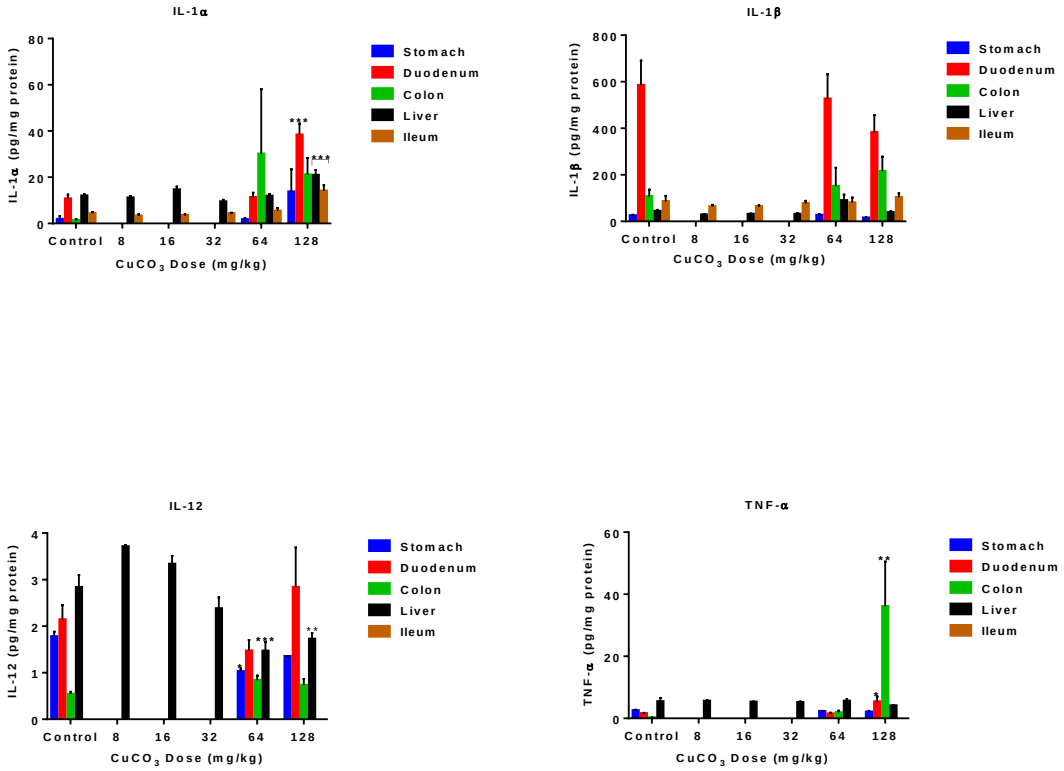


*Figure A. 15. Cytokine concentrations (µg/mg protein) present in selected tissues from rats dosed orally with 8 to 128mg/kg of $CuCO_3$ and sacrificed 6 days post exposure. Data represent the mean±SEM of each cytokine detected (µg/mg protein) and represents data from four separate animals. (*p<0.05; **p<0.01; ***p<0.001 compared with the control).*

GSH and reduced GSH in the liver - There was no significant change in the concentration of both total and reduced GSH in the liver of animals treated orally with different doses of $CuCO_3$.

Conclusions on oral $CuCO_3$ administration.

Oral $CuCO_3$ administration for 5 consecutive days clearly induced toxicity in male rats as indicated by the weight loss of the animals in the highest dose investigated (128 mg/kg). For this dose, it was not possible to maintain the animals for a recovery period of three weeks after the last administration. In addition, to the body weight loss, a reduction in organ weight was noted for the heart, liver, spleen, thymus and an increase for the adrenals. At one dose lower (64 mg/kg) such weight alterations were not present. An increase in WBC was observed that could be attributed to an absolute increase of neutrophilic granulocytes. While the relative percentage of lymphocytes was decreased, the absolute number of lymphocytes remained

similar to control levels. Also for red blood cell parameters (RBC, HgB and Hct) increase was observed. After a recovery period of 21 days these effects were no longer visible. The clinical chemistry data showed alterations in various liver enzymes including ALT, AST, ALP, while the increase in LDH may indicate cell damage. Most of these clinical chemistry parameters returned to normal values after the recovery period of 21 days.

The histopathology confirmed the severe toxicity of the 5-day oral administration of $CuCO_3$. There were severe pathological lesions in the GI-tract with inflammation in the stomach, and cell death (apoptosis) in duodenum and ileum, and ulcerative lesions/erosions in the large intestines (cecum, colon, rectum). The liver showed inflammation, hepatocellular hypertrophy, hepatocellular necrosis and single cell necrosis, especially in the two high dose groups (64 mg/kg and 128 mg/kg). In the lymphoid organs spleen and thymus, severe lymphocyte depletion was present. In contrast in the bone marrow, an increase in myeloid white blood cell precursors was present, which may be present as a compensatory mechanism for the lymphoid depletion in spleen and thymus. In the kidney tubular necrosis, a tubular degeneration/regeneration was observed.

In a number of affected organs, the Cu measured was increased compared to the values in control animals including liver, kidney, spleen, and thymus. Other organs showing an increase in Cu content are lung, mesenteric lymph node testes and brain. However, the increases in testes and brain are limited.